INVESTIGATING THE EFFECTS OF EXPRESSING APE1 HUMAN POPULATION VARIANTS IN CELLULAR SYSTEMS

by

Conan Ma

B.Sc., University of Northern British Columbia, 2009

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
MATHEMATICAL, COMPUTER AND PHYSICAL SCIENCES
(CHEMISTRY)

THE UNIVERSITY OF NORTHERN BRITISH COLUMBIA

April 2012

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-87536-0

Our file Notre référence
ISBN: 978-0-494-87536-0

Abstract

Apurinic/apyrimidinic endonuclease 1 (APE1) is a multi-functional mammalian protein which has recently been shown to possess the ability to endonucleolytically cleave single-stranded RNA and abasic RNA. Several population variants of APE1 (L104R, E126D and D148E) are known to exist in the human population. L104R and E126D have been linked to Amyotrophic Lateral Sclerosis while D148E has been linked to various cancers. The exact molecular mechanisms which correlate these variants with human diseases are currently unknown. Recent evidence has shown that the *in vitro* endoribonuclease activities of these variants are different from the wild-type APE1 protein. Here, we hypothesize that the altered endoribonuclease activity of APE1 population variants may be associated with phenotype changes leading to disease pathogenesis. The goal of this thesis was to determine whether APE1 population variants can cause an altered phenotype when expressed in prokaryotic (Origami™ (DE3) cells) and eukaryotic systems (HeLa cervical cancer and HepG2 hepatoma cancer cell lines). Subsequently, these changes were to be linked to altered endoribonuclease activity of these variants.

Using two separate assays, it was shown that the L104R and E126D variants possess enhanced cytotoxicity to Origami™ (DE3) cells. This correlates with their distinct endoribonuclease activity demonstrated *in vitro*. The D148E variant, which had lost endoribonuclease activity, had no effect on colony formation and growth of Origami™ (DE3) cells. Interestingly, this study also showed that, when over-expressed, the L104R and E126D variants are capable of causing enhanced growth in the mammalian HepG2 cells. Preliminary microarray and quantitative real time polymerase chain reaction experiments were conducted in an attempt to understand the mechanism for the L104R-induced cell growth in HepG2 cells.

Unfortunately, the results were inconclusive. In summary, this thesis has demonstrated a solid correlation between having distinct endoribonuclease activity and the ability to influence cell growth in prokaryotic and eukaryotic cells such as shown for the L104R and E126D APE1 population variants. Such observations provide a framework for future investigations into determining how changes in endoribonuclease activity may be linked to human diseases.

Table of Contents

Chapter 1 – Introduction

Chapter 2 – A Prokaryotic System in Assessing the Ribonuclease Activity of Human Population Variants of APE1

Chapter 3 – Eukaryotic Systems in Assessing the Effects of Over-Expressing APE1 Population Variants

List of Tables

List of Figures

Acknowledgements

First and foremost, I would like express my gratitude to my MSc. supervisor, Dr. Chow H. Lee, for providing the opportunity for me to pursue this degree. Your patient guidance was crucial in the completion of this research project. I would also like to acknowledge my supervisory committee members, Drs. Andrea Gorell and Sarah Gray, for their insightful advice and support. Next, I would like to thank several members of the Northern Biosciences Research Unit. The first is Dr. Maggie Li for her technical support in the laboratory. Everyone else, in no particular order, for their kind friendship: Joseph, Mavis, Eunice, Navkaran, Manbir, Dustin, Daljeet, Liz, Chris, the other Chris, the other other Chris, Dana, Randi, Mark, Justin, Garrett, Shirley, Jennifer, Travis, Carly, Matt, Ben, Michael and Jordie. Finally, I would like to thank my parents for providing me with encouragement throughout all my studies while at university.

Candidate's Publications Relevant to this Thesis

Articles

Kim, W.C., Ma, C., Kim, S-E., Li, W.M., Wilson, D.M. III., and Lee, C. (2012). Distinct Endoribonuclease Function of Apurinic/Apyrimidinic Endonuclease 1 Variants Identified in the Human Population. *Manuscript in preparation for submission.*

Abstracts

Ma, C., Kim, W.C., Li, W.M and Lee, C.H. (2011) Investigating the Distinct RNA-Cleaving Functions of APE1 Human Variants. RiboWest Conference, Prince George BC.

Chapter 1

Introduction

In 2009, the Human Apurinic/Apyrimidinic endonuclease 1 (APE1) protein was identified as an endoribonuclease capable of regulating *c-myc* messenger (mRNA) levels in eukaryotic cells. The regulation of mRNA stability is a complex process and is based on many *cis*-regulatory elements and *trans*- acting factors. mRNA stability is recognized as a major point in the regulation of gene expression. Deregulation of the process can lead to disease in eukaryotic organisms. As such, APE1 may be implicated in mammalian diseases. In addition, APE1 genes are known to exist as various single nucleotide polymorphisms (SNPs) in the human population. This can result in amino acid substitutions and the subsequent expression of the human population variants of the APE1 protein. Preliminary studies indicate both possible alterations in mRNA cleavage activity for these variants and correlations between expression of these variants of APE1 and disease. Thus, the primary focus of this thesis is to examine if the expression of these population variants results in altered phenotype as compared to the wild-type APE1 for both prokaryotic and eukaryotic systems. This may indicate a role in possible downstream disease pathogenesis. If these effects are observed, further investigations into the possible molecular mechanism for these altered phenotypes can be pursued.

1.1 Eukaryotic Gene expression and mRNA decay

The regulation of gene expression is a highly complex process which occurs at many levels including transcriptional, post-transcriptional, translational and post-translational (Parker *et al.* 2004). At the post-transcriptional level, one mechanism has been recently identified as a

key control point in gene expression: the regulation of mRNA stability. The mechanism is also sometimes known as mRNA turnover or degradation (Garneau *et al.* 2007). mRNA stability is a term which describes the factors and processes which are involved in the regulation of the half-life of mRNA molecules. The following is a brief description of the importance of mRNA stability in gene expression and the key players involved in the control of mRNA stability.

1.1.1 Significance of mRNA stability in the Control of Gene Expression

The regulation of mRNA stability and degradation has been identified to be present in nearly all organisms (Nicholson *et al.* 1999; Gutierrez *et al.* 1999; Mello *et al.* 2004). Degradation will have a direct effect on the stability of a particular species of mRNA in a cell and will determine the levels of mRNA for that particular gene. The alteration in mRNA levels can result in changes in associated cellular protein levels and have consequent effects on that organism (Ross *et al.* 1995). The degradation of mRNA molecules has been investigated in more detail as evidence has been discovered which implies that mRNA degradation plays a vital role in gene expression. For example, mRNA degradation has been shown to be integral in post-transcriptional gene silencing, also known as RNA-mediated interference. In RNA-mediated interference, specific mRNA molecules are targeted for degradation by complimentary RNA molecules and are subsequently degraded by cellular degradation machinery, resulting in lowered levels of expression of the targeted genes (Belostotsky *et al.* 2004). RNA-mediated interference has been identified as an anti-viral defense mechanism in eukaryotes (Mello *et al.* 2004). Increased stability of an mRNA species can result in a longer half-life for the mRNA molecule with a subsequent increase in protein levels. For example, studies using cDNA arrays in activated T-cells and UV irradiated cells have clearly demonstrated an increase in mRNA levels for genes implicated in vital cellular processes such as the immune response (Raghavan *et*

al. 2002) and stress responses (Fan *et al.* 2002). Further evidence highlights this importance by indicating that mRNA turnover is vital in the proper development of eukaryotic organisms such as *Drosophila melanogaster* by regulating the proper chronological expression of developmental proteins (Thomsen *et al.* 2010). Deregulation of mRNA turnover is associated with the development of cancers, such as leukemia, by causing normal cells to acquire new protein expression profiles conducive to a cancerous phenotype (Audic *et al.* 2004, Steinman *et al.* 2007). As such, mRNA stability and turnover are key control points in cellular function.

1.1.2 General Overview of Factors involved in mRNA stability

There are two known modes of mRNA decay in mammalian cells – either beginning from the ends of the mRNA transcript (exoribonucleolytic fashion) or from an internal site within the mRNA transcript (endoribonucleolytic fashion). Several factors are known to affect the regulation of mRNA stability and degradation. These factors can be categorized into two divisions – *cis*-regulatory elements, consisting of elements of the mRNA molecule itself, and *trans* acting factors, consisting of various protein factors and enzymes. Examples of *cis*-regulatory elements include the 7' methylguanosine cap (m^7G) and 3' poly adenosine tail which both confer a protective effect on mRNA molecules (Garneau *et al.* 2007). Both of these elements must be removed prior to degradation by the conventional exoribonucleolytic pathways, which degrade mRNA molecules from either the 5' or 3' ends. Examples of such *trans* acting factors are generally RNA-binding proteins and various types of enzymes. RNA-binding proteins are proteins which bind to the mRNA molecule, either in the untranslated regions or in the protein coding region. These binding proteins can act to facilitate degradation of the mRNA molecule by recruiting enzymes involved in mRNA decay such as decapping enzymes and ribonucleases. RNA binding proteins can also act to increase the half-life of the

mRNA by blocking the effects of mRNA degrading machinery in the cell and thus subsequently increasing the mRNA levels. An example of this is the Coding Region Determinant-Binding Protein (CRD-BP) (Ioannidis *et al.* 2005).

Another class of proteins involved in the degradation of mRNA are the decapping enzymes. These decapping enzymes are required for removal of the m^7G cap present on mRNA molecules for 5' to 3' degradation of RNA. In mammals, the proteins involved in this process are the mRNA-decapping enzyme subunit 1 (Dcp1p) and mRNA-decapping enzyme subunit 2 (Dcp2p) proteins (Garneau *et al.* 2007). Similarly, removal of the 3' protective poly (A) tail can facilitate the degradation of mRNA molecules in an exoribonucleolytic fashion from the 3' end of the molecule. The proteins involved in this activity, in mammals, are the dual function proteins Poly A Ribonuclease (PARN) and Carbon Catabolite Repressor Protein 4 homolog B (CCR4b-NOT) (Garneau *et al.* 2007).

The final class of enzymes which play a critical role in mRNA stability in cells are the ribonucleases. These enzymes are directly responsible for degradation of the mRNA molecule by hydrolytic cleavage. These can act either from the 5' or 3' ends of the mRNA molecule (referred to as exoribonucleases) or from an internal point in the mRNA molecule (referred to as endoribonucleases). Exoribonucleases are well studied in mammalian cells and were historically considered the primary enzymes responsible for the degradation of mRNA transcripts (Garneau *et al.* 2007). Exoribonucleases can be categorized into 6 superfamilies based on nucleic acid sequence and catalytic properties (Zuo *et al.* 2001). Specific examples of a 5' to 3' exoribonucleases are 5'-3' exoribonuclease 1 (XRN1) (Mitchell *et al.* 1997) and the exosome complex (Lebreton *et al.* 2008). A specific example of a 3' to 5' exoribonuclease is the previously mentioned PARN protein (Martinez *et al.* 2001). Endoribonucleases have been much

less studied in mammalian cells; however, evidence from the last decade suggests that endoribonucleases may play a more important role in gene expression than previously thought due to a potential increase in efficiency for mRNA cleavage unique to the mechanism of endoribonucleolytic cleavage.

1.2 Eukaryotic Endoribonucleases

Historically, the majority of mRNA decay was thought to be carried out in an exoribonucleolytic fashion in which the mRNA molecule is degraded from the 5' end, following decapping, or from the 3' end, following deadenylation (Houseley *et al.* 2009). However, the mechanism of function for endoribonucleases implies that they can bypass both decapping and deadenylation steps and can, in theory, be much more efficient than exoribonucleases.

1.2.1 Significance of Endonucleolytic Cleavage

In the past 5 years, there have been several studies on endoribonucleases which indicate that these enzymes play a more important role in mRNA degradation than previously thought. One example where endoribonuclease activity has been shown to be more important is the exosome. A component of the exosome, Exosome complex exonuclease DIS3 (Dis3/Rrp44), possesses endoribonuclease activity (Lebreton *et al.* 2008). The exosome is known to facilitate the degradation of many mammalian mRNAs and is a vital component of mRNA metabolism where, previously, it was thought Dis3/Rrp44 degraded mRNA exclusively in an exoribonuclelytic fashion (Schaeffer *et al.* 2009). Another example is that endoribonucleolytic cleavage is now known to be associated with nonsense-mediated decay. Nonsense-mediated decay is a vital cellular mechanism used to detect nonsense mutations and is used to prevent the expression of faulty proteins (Chang *et al.* 2007). This process was traditionally thought to occur

only in an exoribonucleolytic fashion (Gatfield *et al.* 2004); however, SMG6, a metazoan-specific nonsense-mediated decay factor, is now recognized to be the catalytic endonuclease responsible for endoribonucleolytic cleavage of nonsense mRNA molecules (Eberle *et al.* 2009; Huntzinger *et al.* 2008). In addition, transcriptome-wide profiling of mammalian mRNA cleavage products demonstrated that there are a large number of endoribonucleolytic cleavage sites for mammalian mRNAs (Karginov *et al.* 2010; Bracken *et al.* 2011; Mercer *et al.* 2011). These cleavage sites are independent of Argonaute2, Drosha and miRNA-directed cleavage, suggesting that endoribonucleoytic cleavage is more wide spread in RNA metabolism than previously thought.

1.2.2 Known Eukaryotic Endoribonucleases

The scientific field studying ribonucleases have identified several eukaryotic endoribonucleases. The number of known endoribonucleases is much lower than that of exoribonucleases and may be a direct result of the prior lack of scientific attention given to these enzymes. The endoribonucleases are generally multi-functional proteins and possess a wide variety of functions in the cell. Some of the endoribonucleases do not have known molecular targets nor are their catalytic mechanisms known. The endoribonucleases that have been studied have varied target RNA substrates and catalytic mechanisms and possess many distinct functions in the eukaryotic cell (Li *et al.* 2010; Tomeki *et al.* 2010).

Several identified endoribonucleases have weak evidence supporting their endoribonuclease activity. These endoribonucleases have no direct evidence for eukaryotic RNA cleavage, have no known cellular targets, or have no known catalytic site or mechanism. The eukaryotic protein ADP-ribosylation factor domain protein 1 (ARD-1) was found to duplicate

RNase E function in prokaryotes (Wang *et al.* 1994); however, there have not yet been any studies demonstrating endoribonuclease activity for this enzyme in eukaryotic cells. Aldolase C possesses a potential molecular target in the human low molecular weight neurofilament (NFL) mRNA molecule (Canete-Soler *et al.* 2005); but, the catalytic site for its endoribonuclease activity remains unknown (Stefanizzi *et al.* 2007). Polysomal ribonuclease 1 (PMR1) is a nuclease capable of cleaving mRNA endonucleolytically in the presence of a cofactor protein, heat shock protein 90 (Hsp90) (Peng *et al.* 2008). Although it is capable of RNA cleavage, PMR1 has no known cellular targets and does not have an identified catalytic site as the primary sequence of this protein does not match any known ribonuclease. Placental protein 11 (PP11) is a predicted endoribonuclease which shares its secondary structure with an amphibian endoribonuclease, XendoU (Laneve *et al.* 2008). *In vitro* experiments have shown that this protein is capable of endoribonuclease activity (Laneve *et al.* 2008), but no structural studies have shown eukaryotic RNase activity.

Several of the identified endoribonucleases have distinct functions in eukaryotic cells owing to their endoribonuclease activity. Dicer and Argonaute 2 (AGO2) play roles in the process of post-transcriptional gene-silencing. Dicer processes long double-stranded RNAs or short-hairpin RNAs in the cytoplasm into products which are involved in silencing target genes *via* the RNA-Induced Silencing Complex (RISC) complex (Kim *et al.* 2009). AGO2 is recognized as the catalytic enzyme in the RISC complex, serving to degrade mRNA targeted by siRNAs and microRNAs (Liu *et al.* 2004). Given the role of Dicer and AGO2 in gene silencing, these enzymes are implicated in a large variety of important physiological roles such as cellular development and maintenance (Knight *et al.* 2001; Bernstein *et al.* 2003; Muljo *et al.* 2005). The previously mentioned Dis3/Rrp44 is the catalytic subunit of the exosome and is implicated in

regulating the cellular levels of many RNA species (Lorentzen *et al.* 2008). The PIN domain of Dis3/Rrp44 is thought to be responsible for the endoribonuclease activity (Schneider *et al.* 2009). The nonsense-mediated mRNA decay factor (SMG6) is responsible for catalytic activity for the process of nonsense-mediated decay (Eberle *et al.* 2009). Nonsense-mediated decay is a vital cellular process protecting cells from the expression of non-sense mutation containing mRNA molecules (Behm-Ansmant *et al.* 2007). The eukaryotic protein Angiogenin cleaves ribosomal RNA (rRNA) and transfer RNA (tRNA) (Saxena *et al.* 1992) and is implicated in protein synthesis. Inositol-requiring protein 1 (IRE1) regulates the expression of a protein involved in the unfolded protein response (UPR), X-box-binding protein 1 (XBP1) by splicing the mRNA transcript of XBP1 (Calfon *et al.* 2002).

Of the identified endoribonucleases, only 2 have been identified to target specific mRNA in eukaryotic cells, which results in regulation of the expression of the targeted gene. Deregulation of these enzymes may lead to disease. For example, RNase L acts as a tumour suppressor through its endoribonuclease activity as mice deficient in RNase L have suppressed levels of apoptosis (Zhou *et al.* 1997; Malathi *et al.* 2004). Mutations of RNase L have also been linked with the development of prostate cancer (Silverman 2003). The second ribonuclease is apurinic/apyrimidinic endonuclease 1 (APE1). APE1 possesses endoribonucleolytic activity *in vitro* (Barnes *et al.* 2009) against *c-myc* Coding Region Determinant (CRD) RNA. Currently, the only known cellular target of APE1 is *c-myc* mRNA (Barnes *et al.* 2009) which was demonstrated using knock down experiments in a HeLa human cervical cancer cell line. As *c-myc* is a powerful oncogene, it is possible that the deregulation of the ribonuclease activity of APE1, through amino acid substitution mutations, could play a role in the pathogenesis of cancer.

1.3 Apurinic/Apyrimidinic Endonuclease 1 (APE1)

The APE1 enzyme is a 36 kDa multifunctional protein and is found in the nucleus (Evans *et al.* 2000), cytoplasm (Tell *et al.* 2005) and mitochondria (Chattopadhyay *et al.* 2006). Its current known activities in the cell include: 1) endonuclease activity on abasic DNA (AP-DNA) in the Base Excision Repair (BER) pathway (Evans *et al.* 2000); 2) acting as a redox factor which activates transcription factors of many growth related genes (Evans *et al.* 2000); 3) 3'-5' exonuclease activity in removing deoxyribonucleoside analogues from DNA (Chou *et al.* 2000); 4) endoribonucleolytic activity against mRNA *in vitro* (Barnes *et al.* 2009); and 5) endonuclease activity on abasic RNA (Berquist *et al.* 2008). All of these functions appear to play critical roles in eukaryotic organisms, whether through maintenance of genomic stability or the ability to directly regulate gene expression. The biochemical functions of the APE1 protein are localized to two distinct protein domains. Redox activity is localized to the N-terminal 61 amino acid residues (Evans *et al.* 2000) while the remaining nuclease-related activities are thought to occur in the remaining C-terminal amino acids (Chou *et al.* 2000; Berquist *et al.* 2008; Barnes *et al.* 2009).

1.3.1. Overview of Abasic DNA Cleavage Activity

APE1 plays two major roles in BER pathway of DNA repair. First, it facilitates the proper orientation of the DNA substrate by removing the glycosylase protein from the DNA substrate (Baldwin *et al.* 2006). Second, it catalyzes the rate-limiting reaction of the BER process - the endonucleolytic cleavage of the phosphodiester backbone 5' to the abasic site (Srivastava *et al.* 1998). The C-terminal domain of APE1 has been implicated in this process – specifically, residues 61 through 318 (Fishel *et al.* 2007). Of these residues, several key amino acids have

been identified as playing vital roles in DNA cleavage and binding. The amino acids E96, D210 and H309 are implicated in the actual cleavage mechanism of the abasic site (Gorman *et al.* 1997; Mol *et al.* 2000; Beernink *et al.* 2001) while residue W280 is involved in substrate recognition. This residue achieves substrate recognition by using the aromatic structure of tryptophan and intercalating it with the abasic site of the AP-DNA (Kaneda *et al.* 2006). Residue C99 is implicated in binding of abasic DNA as mutations of this cysteine residue to a serine residue significantly reduced the ability of APE1 to bind AP-DNA *in vitro* (Mantha *et al.* 2008). Magnesium ions are required for AP-DNA cleavage activity and play a key role in the catalytic reaction by stabilizing reaction intermediates though binding to two metal co-ordination sites (Beernink *et al.* 2001). This magnesium ion is mobile and moves from one metal binding site to the other during the catalytic reaction as determined by molecular dynamics simulations (Oezgeun *et al.* 2007). Other residues which have been identified to play a role in abasic DNA cleavage include D60, D90, Y171, N212, D219, D283, D308 and residues (Tell *et al.* 2005)

1.3.2 Possible Association of AP-DNAase Activity with Disease

The role of APE1 and its AP-DNA cleavage in disease is difficult to ascertain. The DNA repair activity of APE1 has traditionally been considered a protective mechanism against diseases by removing deleterious mutations from genome (Fishel *et al.* 2007). However, there is compelling biochemical evidence that APE1 may play a direct role in not only the genesis of tumours but in disease progression and drug resistance. For example, significantly elevated levels of APE1 protein have been observed in several cancerous tissues such as osteocarcoma (Wang *et al.* 2004), cervical (Qing *et al.* 2009), lung (Wang *et al.* 2009), and ovarian cancers (Al-Attar *et al.* 2010). These elevated levels of APE1 protein are associated with both poor prognosis and drug resistance in patients with these cancers (Qing *et al.* 2009; Wang *et al.* 2004;

Wang *et al.* 2009; Al-Attar *et al.* 2010). Furthermore, the reduction of APE1 protein levels *in vitro*, using siRNA targeting methods, renders cancer cells sensitive to DNA damaging anti-cancer therapies such as radiotherapy and chemotherapeutic agents including alkylating agents (Wang *et al.* 2004), anti-metabolites (McNeill *et al.* 2009), methanosulfates (Luo *et al.* 2004) and oxidizing agents such as H_2O_2 (Xiang *et al.* 2008).

Currently, it is difficult to identify if the AP-DNA cleavage activity of APE1 is tumour suppressing or oncogenic. It may be the case that both scenarios are true and that APE1 may simply be one of many interacting genes which modulate the risk of various types of cancers.

1.3.3 Overview of the Redox Activity of APE1

In addition to its vital role in BER, APE1 appears to have another critical function in mammalian cells. This is the ability of APE1 to directly regulate gene expression through activation of various cellular transcription factors by reducing the transcription factors into their active states. Genes regulated by APE1 in this fashion, either directly or through a signalling cascade, regulate cell growth and division. This includes genes such as c-jun (Abate *et al.* 2007), c-fos (Xanthoudakis *et al.* 1992), Glial cell line-derived Neurotrophic Factor (GDNF) (Kim *et al.* 2009), Multi Drug Resistance Gene 1 (MDRG1) (Chattopadhyay *et al.* 2008), nuclear factor kappa-light-chain-enhancer of activated B cells (nf-kB) (Ando *et al.* 2008), Phosphatase and Tensin Homolog (PTEN) (Fantini *et al.* 2008) and Vascular Endothelial Growth Factor (VEGF) (Wang *et al.* 2007). Using siRNA and proteomics, APE1 has been implicated in regulating a wide variety of cellular functions such as cell growth, apoptosis, intracellular redox state, mitochondrial function, and cytoskeletal structure (Vascotto *et al.* 2009). However, as the study showing these functions was carried out using APE1 knock down experiments, it is not entirely

clear if these modulated gene expression levels are due to the redox or endoribonuclease functionalities of APE1.

The N-terminal domain of APE1, consisting of the 61 N-terminal amino acid residues, provides the redox function. Residues 1-61 are unique as they are not found in the primary sequence of other DNA repair enzymes. This N-terminal domain is thought to be able to function independently of the nuclease domain of APE1 for redox activities (Izumi *et al.* 2005). Of the first 61 amino acid residues, the residues E36 through T61 are vital for redox activity (Walker *et al.* 1993). It had been thought that the residue C65 played a major role in redox activity as demonstrated by C65 being required for mouse survivability (Walker *et al.* 1993). However, a 2003 mutagenesis experiment has showed that this residue is not important for redox activity as mice with homozygous cysteine to alanine mutations at this position were able to survive to adulthood in good health (Ordway *et al.* 2003). As such, it appears that the C65 residue is no longer considered vital for redox activity of APE1.

1.3.4 Possible Association of REDOX Activity with Disease

As target genes for the redox activity of APE1 are so vital to cell function, it is likely that perturbation to the regulation of these genes may lead to the deregulation of cell phenotype and subsequently lead to disease pathogenesis. APE1, through its redox function, may act as a tumour suppressor gene by activating the expression of known tumour suppressor genes. An example of a known tumour suppressor gene which is regulated by the redox function of APE1 is p53 (Jayaraman *et al.* 1997). A loss of APE1 redox activity may lead to lowered levels of p53 which in turn could lead to increased levels of oncogenic proteins. However, APE1 may also act as an oncogene by activating the expression of known proto-oncogenic genes or genes related to

cancer drug resistance and cancer progression such as c-jun (Xanthoudakis *et al.* 1992), c-fos (Xanthoudakis *et al.* 1992), NF-κβ (Ando *et al.* 2008), VEGF (Wang *et al.* 2007), PTEN (Fantini *et al.* 2008) and estrogen receptors (Curtis *et al.* 2009). Elevated levels of these proteins in cells could result in cancer pathogenesis or cancerous cells becoming drug resistant.

As in the case with AP-DNA cleavage activity, it is difficult to determine which role APE1 plays in cancer through its redox function. Does it act like a tumour suppressor gene or an oncogene? There is evidence in the literature which point either way (Jayaraman *et al.* 1997; Xanthoudakis *et al.* 1992; Xanthoudakis *et al.* 1992; Ando *et al.* 2008; Wang *et al.* 2007; Fantini *et al.* 2008; Curtis *et al.* 2009).

1.3.5 Overview of the Endoribonuclease Activity of APE1

In 2009, researchers in Dr. Lee's lab purified and identified two endoribonucleases from rat liver polysomes that were capable of cleaving the oncogenic mRNA species, *c-myc, in vitro*. One of these proteins was subsequently identified to be APE1. APE1 was challenged with radio labelled *c-myc* CRD RNA in endoribonuclease assays and was found to preferentially cleave single stranded regions of RNA at UA, CA and UG dinucleotides (Barnes *et al.* 2009). Furthermore, this study identified that APE1 appears to regulate the cellular expression levels of *c-myc* in HeLa cervical cancer cell lines (Barnes *et al.* 2009). APE1 has not been identified to regulate the expression of any other gene *in vitro*.

Some of the residues critical for this mRNA cleavage activity have been identified (Kim *et al.* 2011). These residues are very similar to the essential residues for AP-DNA cleavage activity (E96, H309) (Kim *et al.* 2011), implying a shared binding and catalytic site between the DNA and RNA cleaving activities. However, the actual mechanism of cleavage between DNA

and RNA is not likely to be the same due to the biochemical differences between DNA and RNA. In support of this, Dr. Lee's lab has further demonstrated that the cleavage mechanism of APE1 for RNA uses the 2' carbon hydroxyl group on the sugar ring in catalysis (Kim *et al.* 2009). DNA has no hydroxyl group on the 2' carbon position, and thus, this mechanism of catalysis is impossible for DNA. Also, a 2009 study has demonstrated that an interaction between APE1 and another protein, Nucleophosmin1 (NPM1), enhances abasic DNA cleavage while inhibiting RNA cleavage (Vascotto *et al.* 2009). Together, these facts suggest a different catalytic site for each substrate type.

The critical residues for binding RNA have not yet been fully elucidated; however, a 2009 electrophoretic mobility shift assay (EMSA) study showed that the 33 N-terminal residues of the APE1 protein play a role in the binding of single stranded RNA. Removal of the 33 N-terminal amino acids results in lost ability of the APE1 protein to bind single stranded RNA (Vascotto *et al.* 2009). These results suggest that this region is also important for the binding and catalytic function of APE1 for mRNA.

1.3.6 Possible Association of Endoribonuclease Activity with Disease

As mRNA regulation is now considered a key control point in the regulation of gene expression, and deregulation of these processes have been implicated in diseases, the possible role of the endoribonuclease function of APE1 in disease is compelling (Garneu *et al.* 2007). Deregulation of gene expression is a hallmark of many diseases, especially cancer. Thus, it is tempting to hypothesize that deregulation of gene expression, through loss of function mutations for the mRNAse activity of APE1, may lead to disease. This hypothesis is bolstered by the finding that APE1 appears to keep the cellular expression levels of a potent oncogene, *c-myc,* in

check in HeLa cervical cancer cell lines through its endoribonuclease activity (Barnes *et al.* 2009).

Some data however, indicate that the endoribonuclease activity of APE1 is not responsible for cancer. If APE1 is responsible for keeping oncogene expression levels in check, then the general over-expression (Qing *et al.* 2009; Wang *et al.* 2004) and cytoplasmic localization of wild type APE1 in cancer tissues (Yoo *et al.* 2008; Kelley *et al.* 2001; Di Maso *et al.* 2007) appear to implicate that a mechanism other than endoribonuclease activity is responsible for disease. The complete picture of genes which are controlled by the endoribonuclease activity of APE1 is not currently known and APE1 may, in fact, be negatively regulating an unknown tumour suppressor gene *via* an endoribonucleoytic mechanism.

1.4 Human Population Variants of APE1

Studies have demonstrated that DNA repair genes are frequently subjected to single nucleotide polymorphisms and single amino acid substitutions such as in the case of X-ray Repair Cross-Complementing protein 1 (XRCC1) (Wang *et al.* 2009) and human 8-Oxoguanine DNA N-Glycosylase 1 (hOGG1) (Weiss *et al.* 2005). APE1 is no different: 19 population variants of APE1 have been identified and several of these variants have been examined for perturbations in the biochemical functions of APE1 demonstrating associations with disease.

1.4.1 Known Population Variants of APE1

The following single amino acid substitution variants of APE1 have been identified in the human population: K35Q, G39E, Q51H, G57A, I64V/T, L104R, P112L, E126D, D148E, R237A/C, G241R, D283G, G306A, P311S, T313A and A317V (Wilson *et al.* 2011). Of the listed mutations, K35Q, G39E, Q51H, G57A, I64V/T are located in the redox domain of the 61

N-terminal amino acids (Izumi *et al.* 2005). The remainders of the mutants (L104R, P112L, E126D, D148E, R237A/C, G241R, D283G, G306A, P311S, T313A and A317V) are located in the nuclease domain of APE1 at the C-terminal domain of the protein (Figure 1). The implications are that K35Q, G39E, Q51H, G57A, I64V/T are unlikely to have effects on nuclease function, including ribonuclease activity, whereas the remainder of the population variants may. Figure 1 illustrates the known population variants of APE1 and their locations relative to the functional domains of APE1.

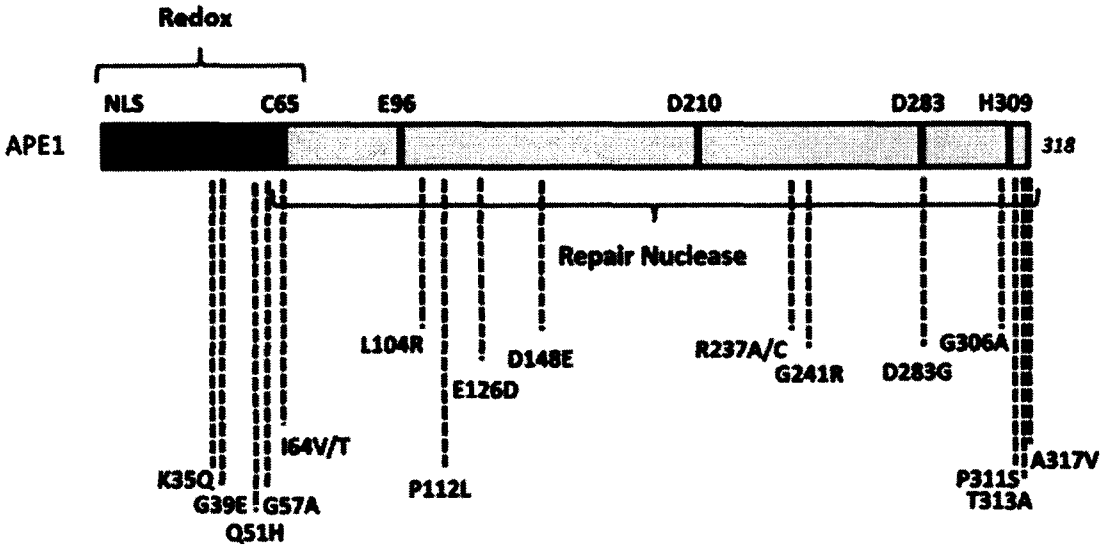

Figure 1. The known human population variants of APE1 and their locations on the APE1 protein relative to the known functional domains of APE1. (Wilson *et al.* 2011)

Two known mutations in the 5' promoter region of the APE1 gene have been also identified. The first mutation is a T→G SNP mutation at nucleotide position 141 (T-141G) which decreases the binding affinity of an APE1 transcription factor for this promoter region resulting in an overall decrease in the expression of APE1 protein levels (Lu *et al.* 2009). A

second mutation is known to occur at position -656 resulting in a SNP of T→G (T-656G). In contrast to the (T-141G) variant, (T-656G) increases cellular levels of APE1 and has thus been demonstrated to increase expression of APE1 (Lo *et al.* 2009).

1.4.2 Known Effects of the Population Variants on APE1 Function

The majority of the population variant studies on APE1 have focused on the effects of the variants on the AP-DNA endonuclease activity. Two variants (Q51H and I64V) are excluded from these studies, as they are located in the redox domain of APE1 and therefore will likely have no effect on nuclease functions of APE1 (Izumi *et al.* 2005). The remainders of the variants have been assayed for their ability to cleave AP-DNA. The variants L104R, E126D and R237A possess a 40-60% reduction in overall AP-DNA cleavage activity as compared to wild-type APE1 (Hadi *et al.* 2000). In contrast, G241R has a 10% increase in AP-DNA cleavage activity (Hadi *et al.* 2000). Both D148E and G306A have identical cleavage ability as compared to wild-type APE1 (Hadi *et al.* 2000).

Experiments have been carried out to examine the effect of these variants on APE1 endoribonuclease activity. When challenged with an 88-nt long *c-myc* RNA substrate, the D148E, R237A, G241R and G306A variants showed decreased RNA cleaving activity as compared to that of wild-type APE1 (Kim *et al.* 2012). The L104R and E126D variants possess altered cleavage sites on the RNA substrate as both variants lost cleavage sites and gained additional unique cleavage sites, respectively, as compared to the cleavage sites of wild-type APE1(Kim *et al.* 2012). In addition to these altered cleavage patterns, these two variants maintained RNAse activity even in the presence of a powerful RNase inhibitor, RNasin (Kim *et al.* 2012). RNasin is a small peptide inhibitor of RNase activity which functions by the

noncovalent bonding of RNasin to RNases (Kim *et al.* 1999). Wild-type APE1 no longer possesses RNase activity in the presence of RNasin. The ability of L104R and E126D to cleave in the presence of RNasin suggests that these variants may be more active in their RNase activity than APE1. A summary of the effects of the population variants on the nuclease functions of APE1 are listed below in Table 1. Population variants not characterized for nuclease activity are not listed.

Table 1. APE1 human population variants and their effects on nuclease activity for AP-DNA and *c-myc* CRD RNA. Reductions in cleavage activity are relative to wild-type APE1.

Mutant	**Effect on AP-DNA Endonuclease Activity**	**Effect on RNA Cleaving Activity**
D148E	No effect (same as wild-type APE1) (Hadi *et al.* 2000)	Lost cleavage activity (Kim *et al.* 2009)
E126D	40-60% reduction in cleavage activity (Hadi *et al.* 2000)	Altered cleavage sites on *c-myc* CRD RNA substrate (Loss of wild-type APE1 clevage sites, addition of novel cleavage sites) (Kim *et al.* 2009)
L104R	40-60% reduction in cleavage activity (Hadi *et al.* 2000)	Altered cleavage pattern (Loss of wild-type APE1 clevage sites, addition of novel cleavage sites) (Kim *et al.* 2009)
R237A	40-60% reduction in cleavage activity (Hadi *et al.* 2000)	Lost cleavage activity (Kim *et al.* 2009)
G241R	Slightly enhanced cleavage activity (Hadi *et al.* 2000)	Lost cleavage activity (Kim *et al.* 2009)
G306A	No effect (same as wild-type APE1) (Hadi *et al.* 2000)	Lost cleavage activity (Kim *et al.* 2009)

1.4.3 Known Associations of the Population Variants of APE1 to Diseases

Several population variants of APE1 have been documented in the literature for association with human diseases. In contrast, some variants appear to confer a protective effect to diseases. Select variants display a correlation with disease as well as a correlation with a protective effect. Despite the large number of studies on the APE1 variants and disease, there are few studies attempting to link these associations with any biochemical function of APE1. The association of the APE1 population variants with disease remains an intriguing issue with no clear mechanism.

There are two documented mutations of APE1 which occur in the 5' promoter region of the gene. In population studies where APE1 was sequenced, the T-141G variant appears to confer protective effects against cancer pathogenesis in lung cancer when compared to individuals carrying the wild-type version of APE1 (Lu *et al.* 2009; Li *et al.* 2011; Zhou *et al.* 2011). In contrast, the T-656G variant increases the risk of lung cancer in similar studies (Lo *et al.* 2009).

The I64V variant was originally identified in a DNA sequencing experiment from lung cancer and healthy tissues from a Norwegian population. Healthy individuals had a statistically higher occurrence of I64V, compared to wild-type APE1, thus implicating a protective effect of this population variant against lung cancer (Zeinolddiny *et al.* 2006). However, when the I64V variant is present in conjunction with an environmental risk factor (vinyl chloride monomer), there is a higher risk of lung cancer as compared wild-type APE1 (Wen-Bin *et al.* 2009).

The L104R and E126D variants were first identified in a screening study for APE1 in relation to Amyotrophic Lateral Sclerosis (ALS). Sequencing of individuals resulted in the

identification of these variants in individuals displaying the disease (Olkowski *et al.* 1998). These findings suggest that the L104R and E126D variants may have a role in the pathogenesis of ALS; however, subsequent studies have shown the absence of both these variants from larger screening experiments, indicating these variants may not be implicated in ALS (Hayward *et al.* 1999; Tomkins *et al.* 2000). No known studies have implicated these variants in cancer.

The majority of studies of APE1 population variants have focused on the D148E variant. This may be due to its high allele frequency in the human population as it is present in roughly 0.380% of the general population with an allele distribution of D/D = 54, D/E = 45 and E/E = 25 (Hadi *et al.* 2000). The D148E variant has been shown to be associated with a wide variety of cancers such as bladder (Narter *et al.* 2008), colon (Pardini *et al.* 2008; Kasahara *et al.* 2008; Canbay *et al.* 2011), lung (Ito *et al.* 2004; Agachan *et al.* 2009; Gangwar *et al.* 2009), pancreatic (Jiao *et al.* 2006), prostate (Chen *et al.* 2008), and stomach cancers (Canbay *et al.* 2011) in various DNA sequencing experiments. The D148E variant is capable of causing an increased risk of cancer for both homo and heterozygous substitutions (Narter *et al.* 2008; Pardini *et al.* 2008; Agachan *et al.* 2009). In addition, the majority of the studies show that the APE1 variant, by itself, is capable of causing an increased risk of cancer (Narter *et al.* 2008; Gangwar *et al.* 2009; Canbay *et al.* 2011). However, some studies demonstrate that the APE1 polymorphism requires associations with polymorphisms of other DNA repair proteins, such as XRCC1 or hOGG1, to facilitate an increase in cancer risk (Jiao *et al.* 2006; Chen *et al.* 2008). Despite the quantity of studies which demonstrate an association of D148E with increased cancer risk, there are almost an equal number of studies demonstrating either no increase in risk or an outright protective effect against various forms of cancer. These studies include cancers such as bladder (Terry *et al.* 2006; Gangwar *et al.* 2009), esophageal (Tse *et al.* 2009), head and neck (Li *et al.* 2007), lung

(Misra *et al.* 2003; Zeinolddiny *et al.* 2006; Chang *et al.* 2009), pancreatic (Li *et al.* 2007), stomach (Li *et al.* 2009; Palli *et al.* 2010) and thyroid cancers (Chiang *et al.* 2008) for this D148E variant.

Known associations of APE1 variants and their effects on several human diseases are contradictory. There is still current research being performed in regards to identifying the relationship of these variants with cancer. However, the D148E variant has been most recently shown to be associated with cancer instead of being shown to be protective against cancer (Canbay *et al.* 2011). Thus, investigation into the biochemical functions of how the APE1 variants may be causing disease is of interest. Table 2 summarizes the APE1 population variants risk and diseases.

Table 2. Human APE1 population mutants and their associations with disease. Risk can be mediated by only the presence of an APE1 polymorphism (denoted as 'alone') or by the combination of APE1 and other DNA repair protein polymorphisms (denoted as 'mixed'). ↑ indicates an increased risk of disease, while ↓ indicates a decreased risk of disease.

Type of Disease	**Variant**				
	D148E		*I64V*	*T-141G*	*T-656G*
Bladder Cancer	↑Risk (Narter *et al.* 2008) (alone) Risk (Gangwar *et al.* 2009) (alone)	No Change (Terry *et al.* 2006)			↓ Risk (Wang *et al.* 2010) (alone)
Colon Cancer	↑ Risk (Paradini *et al.* 2008) (mixed) ↑ Risk (Canbay *et al.* 2011) (alone)	↑ Risk (Kasahara *et al.* 2008) (alone) ↑ Risk (Kasahara *et al.* 2008) (mixed)			
Esophageal Cancer	No Change (Tse *et al.* 2009)				
Head and Neck	No Change (Li *et al.* 2007)				
Lung Cancer	↑ Risk (Ito *et al.* 2004) (mixed)	No Change (Chang *et al.* 2009)	↓ Risk (Zeinolddiny *et al.* 2006) (alone) ↑ Risk (Wen-Bin *et al.* 2009) (mixed – Environmental Toxin - vinyl chloride monomer)	↓ Risk (Lu *et al.* 2009) (alone)	↑ Risk (Lo *et al.* 2009) (mixed)
	↑ Risk (Shen *et al.* 2005) (alone)	No Change (Zeinolddiny *et al.* 2006)		↓ Risk (Li *et al.* 2011) (alone)	
	↑ Risk (Agachan *et al.* 2009) (alone) ↑ Risk (Gangwar et al. 2009) (mixed)	No Change (Misra *et al.* 2003)		↓ Risk (Zhou *et al.* 2011) (alone)	
Pancreatic Cancer	↑ Risk (Jiao *et al.* 2006) (mixed)	↓ Risk (Li *et al.* 2007) (alone)			
Prostate Cancer	↑ Risk (Tse *et al.* 2008) (mixed)				
Skin Cancer	↑ Risk (Breton *et al.* 2007) (alone)				
Stomach Cancer	No Change (Li *et al.* 2009) (alone) ↑ Risk (Canbay et al. 2010) (alone)	No Change (Palli *et al.* 2010) (alone)			
Thyroid Cancer	No Change (Chiang et al. 2008) (alone)				

1.5 Research Objectives

There are numerous pieces of evidence implicating the population variants of APE1 in human diseases. In particular, the D148E variant has been implicated in many types of cancers (Narter *et al.* 2008; Pardini *et al.* 2008; Kasahara *et al.* 2008; Canbay *et al.* 2011; Jiao *et al.* 2006; Tse *et al.* 2008; Canbay *et al.* 2011) while the L104R and E126D variants have been shown to be associated with ALS (Olkowski *et al.* 1998). It is hypothesized that these variants cause a change in the biochemical activity of APE1 which leads to downstream cellular changes and thus disease. However, the exact biochemical function of APE1 that is being affected which ultimately leads to disease by these variants remain unknown at this time. Previous work has identified the variants to have full or modestly reduced AP-DNA cleavage activity as compared to the wild-type APE1, suggesting that the DNA endonuclease activity of APE1 is not likely responsible for disease (Hadi *et al.* 2000). In conjunction with the AP-DNA activity, the RNA cleaving activity of D148E is lost while the L104R and E126D variants have altered RNA cleavage patterns as compared to wild-type APE1 (Kim *et al.* 2012). Given that the AP-DNA activity of D148E, L104R and E126D variants was not significantly altered (Hadi *et al.* 2000), we hypothesize that the link between these variants and human disease may be explained by their significantly altered ribonuclease activity (Kim *et al.* 2012). This forms the basis for the research objectives of this thesis.

The first objective of this thesis was to demonstrate if the population variants of APE1, when expressed in a prokaryotic system, would cause an altered phenotype (in this case, growth) for the cells when compared to wild-type APE1 expression. The system used was the Origami™ (DE3) cells. Due to mutations for cellular reductases, these Origami™ (DE3) cells allow for proper folding of proteins and formations of structurally important cysteine bonds. For instance,

proper folding of ribonucleases in Origami™ (DE3) cells results in cellular death (Smith *et al.* 2006). Wild-type APE1 and the population variants were expressed in Origmai™ cells and the subsequent levels of cytotoxicity were observed. The results were expected to answer two questions: 1) Are the previously observed *in vitro* RNA cleaving activity relevant in a cellular system?; 2) Is there a correlation between ribonucleolytic activity and cell cytotoxicity in Origami™ (DE3) cells exerted by APE1 and its human population variants?

The second objective arises from the results of the first objective. Here, the primary objective was to demonstrate if the population variants, when expressed in mammalian cells, would cause an altered phenotype upon expression as compared to the wild-type APE1 expression. Two cell lines were chosen for the thesis experiments, HeLa cervical cancer cells and HepG2 liver cancer cells. APE1 and its population variants were expressed in these cells and the subsequent effect on growth rate were measured using a standard MTT growth assay. The results were expected to give insight into whether the population variants of APE1 are relevant in mammalian systems for causing phenotypic changes and thus possibly leading to downstream disease.

The third objective of this thesis was to investigate if the population variants of APE1 caused alterations in the mRNA levels of specific genes leading to growth rate changes in mammalian cells as measured by MTT assays. APE1 and its population variants were expressed in mammalian cells at which point total RNA was harvested and subjected to microarray analysis. Given the preliminary microarray data, real time quantitative polymerase chain reaction analysis was then used to examine alterations of mRNA levels of selected genes upon over-expression of APE1 and its population variants. The results were expected to demonstrate if APE1 causes phenotypic changes in mammalian cells owing to its endoribonuclease activity.

Chapter 2

A Prokaryotic System in Assessing the Ribonuclease activity of Human Population Variants of APE1

2.1 Introduction

This chapter is dedicated to the experimental approaches used to investigate the cellular cytotoxicity contributed by the ribonucleolytic activity of APE1 and its population variants in a prokaryotic system, Origami™ (DE3) cells. In addition, this section will cover data presentation and analysis as well as implications in mammalian systems.

In 2009, APE1 was identified as an endoribonuclease capable of cleaving *c-myc* CRD RNA *in vitro* and regulating *c-myc* mRNA levels in HeLa cells (Barnes *et al.* 2009). The finding could place APE1 as having a role in the pathogenesis of diseases as mRNA turnover is identified as a key control point in gene expression for eukaryotes (Garneau *et al.* 2007). In addition, several population variants of APE1 exist (Wilson *et al.* 2011), and some of these variants demonstrate altered RNA cleavage *in vitro* (Kim *et al.* 2012). It is possible that these *in vitro* findings could translate to the variants possessing altered ribonuclease function in cellular systems, and possibly to pathogenesis in organisms. Indeed, mutations to an endoribonuclease known as RNase L have been shown to play a role in the development of prostate cancer (Silverman 2003). However, before investigation of the APE1 variants in a mammalian system could be performed, it was deemed appropriate to determine if these variants have any cellular effect in a simple and more easily manageable biological system.

The system chosen for analysis in prokaryotic cells was the Origami™ (DE3) cells. These cells are a strain of *E. coli* with specific mutations to their thioredoxin reductase and glutathione reductase genes (Smith *et al.* 2006). These mutations result in an oxidizing cytoplasm conducive for disulfide bond formation for any exogenously introduced protein *via* plasmid transformation (Smith *et al.* 2006). In the case of ribonucleases, proper folding of the exogenous protein will result in measureable cytotoxicity of the Origami™ (DE3) cells, ostensibly due to RNA cleavage. Origami™ (DE3) cells have been used to demonstrate the RNA cleaving function of the endoribonucleases RNase A (Smith *et al.* 2006) and Angiogenin (Smith *et al.* 2008).

Two separate assays were performed: 1) a transformation efficiency based plate protocol; and 2) a transformation efficiency independent liquid broth protocol. The population variants analyzed in detail were the Q51H, I64V, L104R, E126D, D148E and G306A APE1 variants.

2.2 Methodology

2.2.1 Preparation of pET15b-APE1 Constructs

A plasmid construct consisting of the vector pET15b and the APE1 cDNA containing a Q51H mutation was donated to our lab by Dr. Sankar Mitra from the University of Texas Medical Branch at Galveston. From this, our lab generated the pET15b-APE1 wild-type plasmid construct as well as the pET15b-I64V and pET15b-D148E population variants *via* site directed mutagenesis (Kim *et al.* 2009). The pET15b-APE1 construct was subsequently used to generate the remaining population variant constructs (L104R, E126D, and G306A) using a PCR-based site directed mutagenesis protocol. Table 3 lists the primers used for the site-directed mutagenesis. A 50 µl reaction mixture was prepared containing 100 ng of pET15b-APE1

(template), 3.6 mM dNTPs, 1.1 μM forward primer, 1.1 μM reverse primer, 1X Phusion DNA polymerase buffer and 0.04 units of Phusion DNA polymerase (Finnzymes Scientific). The PCR reaction was carried out in an MJ Research MiniCycler using the following PCR cycle: Denaturation at 95°C for 45 seconds, primer annealing at 55°C for 45 seconds and extension at 68°C for 10 minutes for a total of 18 cycles. This was followed by a final 20 minute extension step at 68°C and storage at 4°C. DNA sequencing by Macrogen Inc. (Seoul, South Korea) was used to confirm the identity of the population variant pET-15b vectors.

Table 3. List and sequence identity of primers used for generation of population variant pET-15b plasmid constructs. Highlighted nucleotides are responsible for the amino acid substitution.

Plasmid Construct	Forward Primer	Reverse Primer
L104R	5'-TCA GAG AAC AAA CGA CCA GCT GAA CTT-3'	5'-AAG TTC AGC TGG TCG TTT GTT CTC TGA-3'
E126D	5'-CCT TCG GAC AAG GAC GGG TAC AGT GGC-3'	5'-GCC ACT GTA CCC GTC CTT GTC CGA AGG-3'
G306A	5'-TCC AAG GCC CTC GCC AGT GAT CAC TGT-3'	5'-ACA GTG ATC ACT GGC GAG GGC CTT GGA-3

2.2.2 Preparation of Competent Origami™ (DE3) Cells

Origami™ (DE3) cells were acquired from Novagen (Germany). Origami™ (DE3) cells contain resistance genes towards the kanamycin and puromycin antibiotics. For selection purposes, all plates and liquid cultures used for culturing Origami™ (DE3) cells contained 12.5 μg/ml of kanamycin and 100 μg/ml of puromycin. Cells were streak plated and grown overnight at 37°C. Single colonies were selected and cultured overnight in 10 mL cultures at 37°C by shaking at 200RPM in an Innova40™ shaker. Five ml of these overnight cultures were then subcultured into 500 ml cultures and grown at 37°C and 200 RPM until the optical cell density

(OD_{600}) was determined to approach 0.400 absorbance units using a DU 800 UV/visible spectrophotometer. Cells were spun down in an Allegra X-12R Centrifuge for 30 minutes at 3500 RPM and re-suspended in 30 ml cold $CaCl_2$ for 30 minutes. Cells were re-centrifuged at 3500 RPM for 120 minutes and re-suspended in 4 ml of $CaCl_2$ solution containing 15% glycerol. Cells were split into 100 µl aliquots and kept at -80°C until use.

2.2.3 Preparation of LB-Ampicillin Plates

Agar containing Luria Broth (LB) was melted using a microwave. Upon liquefaction, ampicillin was added to the LB-agar to a final concentration of 100 µg/ml. 15 ml of the LB-Amp was then aliquoted into 100 mm Petri dishes (Fisherbrand, UK) and allowed to solidify at room temperature. Plates were then stored at 4°C until used.

2.2.4 Plate Protocol for Assessment of Cytotoxicity of APE1 and its Population Variants in Origami™ (DE3) Cells

pGEX4T3-RNase A vector construct was generously gifted for use in this experiment by Dr. Ronald Raines, University of Wisconsin-Madison. 100 ng of pGEX4T3-RNase A, empty vector pET15b, pET15B-APE1 or pET15B containing the population variants of APE1 (pET15B-Q51H, pET15B-I64V, pET15B-L104R, pET15B-E126D, pET15B-D148E, pET15B-G306A) were pipetted to single aliquots of Origami™ (DE3) cells and incubated on ice for 30 minutes. The cells were then transformed *via* heat shock at 42°C for 60 to 75 seconds and expanded with 1 ml of LB broth. After thorough mixing, 150 µl was then then plated onto ampicillin plates (100 µg/ml) partitioned into thirds for comparison of transformation conditions. Plates were left to incubate overnight at 37°C and the resulting colonies for each transformation condition were counted. Images were acquired using the Alpha Innotech ChemiImager and

FluroChem 5500 software. Data from 3-5 biological replicates were performed for each transformation. Statistical analysis were performed by normalizing the number of surviving colonies of each of the population variant transformed cells to that of wild-type APE1-transformed cells and then performing a one way analysis of variance (ANOVA) test.

In addition to the 100 ng transformation previously carried out, transformations using 300 ng and 500 ng of empty pET15b, pET15b-APE1, pET15b-L104R, and pET15b-E126D plasmid constructs were performed. Statistical analyses were performed using a one way ANOVA test as previously described.

2.2.5 Broth Protocol for Assessment of Cytotoxicity of APE1 and its Population Variants in Origami™ (DE3) Cells

Individual Origami™ (DE3) colonies containing empty pET15-b vector or pET15b-APE1 constructs (pET15b-APE1, pET15b-Q51H, pET15b-I64V, pET15b-L104R, pET15b-E126D, pET15b-D148E, pET15b-G306A) were prepared. Single 100 ml aliquots of cells were transformed with 100 ng of plasmid and subsequently heat shocked at 42°C for 1 minute. Upon transformation, cells were plated on LB-Ampicillin (100 μg/ml) plates and grown overnight. Individual colonies of each plasmid transformed cell type were then selected and picked using a sterile loop and transferred to 1 ml of LB-Ampicillin broth (100 μg/ml). The cultures were then grown for one hour at 37°C. The cell counts for each of the plasmid transformed cell types were then determined using a previously established standard curve for *E. coli* cell density at OD_{600}.

$$\text{Cell Count} = 1.0^{9}(OD_{600})^{3.3158}$$

All plasmid transformed cell types were then equalized to a common cell count using LB-Ampicillin broth and subsequently transferred to 96-well tissue culture plates in triplicates. The

initial OD_{600} was measured for each plasmid transformed cell type and recorded as the initial cell count at time point zero. The culture plates were then placed in an Innova40™ incubator shaker set at 37°C and 200 rpm to facilitate cell growth. The tissue culture plates were removed from the incubator and the OD_{600} taken for each sample type every hour after the initial reading for a total of 12-15 hours. The data obtained were plotted as growth curves in Kaleidagraph 4.0™ (Synergy Software, USA). Subsequently, the generation time for each transformation was also determined using KaleidaGraph 4.0™ (Synergy Software, USA) using the equation fit parameter

$$e^{((0.693147/m1)*m0)*m2;m1=5;m2=0.05}$$

which describes doubling time of bacterial cells during the exponential growth phase where the m variables describe the shape, starting and end points of the fitted curve. The data collected from 3-5 biological replicates were then pooled and standard deviation was calculated.

2.3 Results

To determine if the plate assay was suitable for detecting differences in cellular cytotoxicity, and by extension ribonucleolytic activity, a control plate was performed using pET15b-Empty vector as a negative control and pGEX4T34T3-RNase A as a positive control. Previously, transformation of pGEX4T34T3-RNase A had been demonstrated to be completely cytotoxic to cells and resulted in no surviving colonies (Raines *et al.* 2006). A plasmid dosage of 300 ng was chosen as the initial plasmid concentration since previous studies in our lab had demonstrated that this amount of plasmid was suitable for the study of APE1 in Origami™ (DE3) cells. Here, the empty vector pET15b-transformed Origami™ (DE3) cells produced many colonies (Figure 2A, section 1), consistent with no introduced exogenous protein. In contrast, the pGEX4T3-RNase A-transformed cells resulted in total cell death and no resultant surviving

colonies due to the expression of RNase A (Figure 2A, section 3). As an intermediary condition, the pET15b-APE1-transformed cells produced fewer colonies than the empty-vector-transformed-cells but more colonies than the pGEX4T3-RNase A-transformed cells (Figure 2A, section 2). Thus, there was a discernible sensitivity in detecting increases in cytotoxicity between condition types; here we see cytotoxicity of wild-type APE1 but to a lesser degree than that of RNase A. Therefore, this assay was deemed suitable for determining possible differences in cytotoxicity between wild-type APE1 and its population variants.

Origami ™ (DE3) cells transformed with the plasmid pET15b-Q51H showed an equal number of surviving colonies compared to wild-type APE1 transformation (Figure 2B, section 1 versus section 2). This was again observed for the I64V (Figure 2B, section 3 versus section 2), D148E (Figure 2D section 1 versus section 3), and G306A variants (Figure 2D section 1 versus section 3). At least 3 biological replicates were performed for each plasmid type. ANOVA analysis confirmed that the Q51H, I64V, D148E and G306A-transformed cells did not produce a statistically significant decrease in surviving colonies as compared to wild-type APE1-transformed cells (Figure 4). In contrast, both the L104R and E126D variants resulted in 25% and 35% surviving colonies as compared to the wild-type APE1, respectively (Figure 2C, section 1 versus section 2 and Figure 2C, section 3 versus section 2, respectively). At least 3-5 biological replicates were performed for these variants, and ANOVA analyses confirmed that the L104R and E126D transformations were more cytotoxic to Origami™ (DE3) cells than the wild-type APE1 transformations ($P < 0.0001$) (Figure 4).

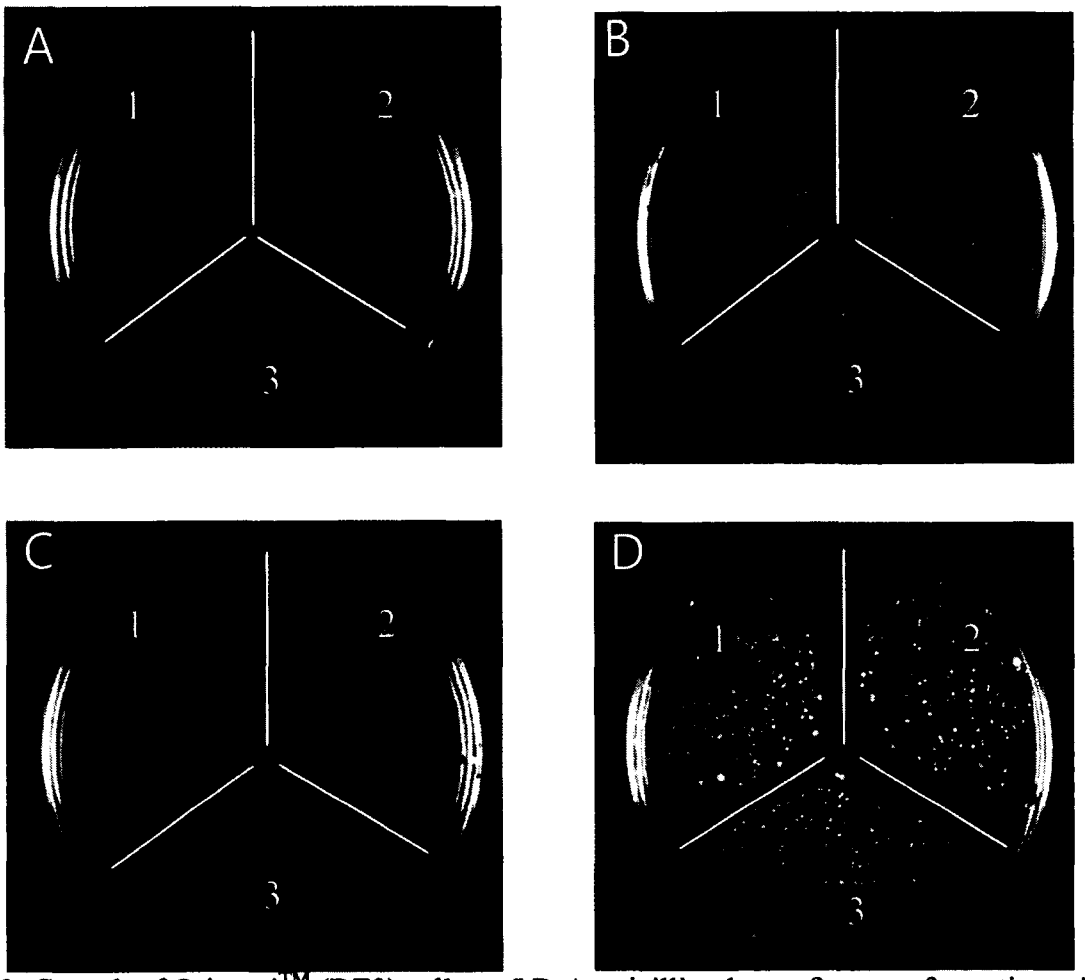

Figure 2. Growth of Origami™ (DE3) cells on LB-Ampicillin plates after transformation with various pET15b plasmid constructs. Plates were incubated at 37°C for 24 hours. 300 ng plasmid of:
A: 1) empty vector pet15b; 2) pET15B-APE1; 3) RNase A pGEX4T3
B: 1) pET15b-Q51H; 2) pET15B-APE1; 3) pET15b-I64V
C: 1) pET15b-L104R; 2) pET15B-APE1; 3) pET15b-E126D
D: 1) pET15b-D148E; 2) pET15B-APE1; 3) pET15b-G306A

To further investigate the mechanism for this observed increase in cytotoxicity, an experiment was devised to test for various concentrations of plasmid transformation (100 ng, 300 ng and 500 ng) (Figure 3). Each transformation condition was performed in biological triplicates. For 100 ng plasmid transformation, L104R and E126D-transformed cells showed 25% and 35% surviving colonies compared to that of the wild-type APE1, respectively (Figure 3A, section 1 versus section 2 and Figure 3A section 3 versus section 2 respectively). One way ANOVA shows

that this difference is significant ($P < 0.0001$) (Figure 4). This trend held true for the 300 ng transformation (Figure 3B, section 1 versus section 2 and Figure 3B section 3 versus section 2, respectively) where the L104R transformation resulted in 25% surviving colonies and the E126D transformation resulted in 30% surviving colonies as compared to the wild-type APE1 transformations (Figure 4). One way ANOVA shows that this difference is significant ($P < 0.0001$) (Figure 4). However, for the 500 ng transformations, the L104R transformation no longer showed any significant difference in surviving colonies as compared to the wild-type APE1 transformation (Figure 3C, section 1 versus section 2) while the E126D transformation still showed a decreased number of colonies as compared to wild-type APE1 (Figure 3C, section 3 versus section 2).

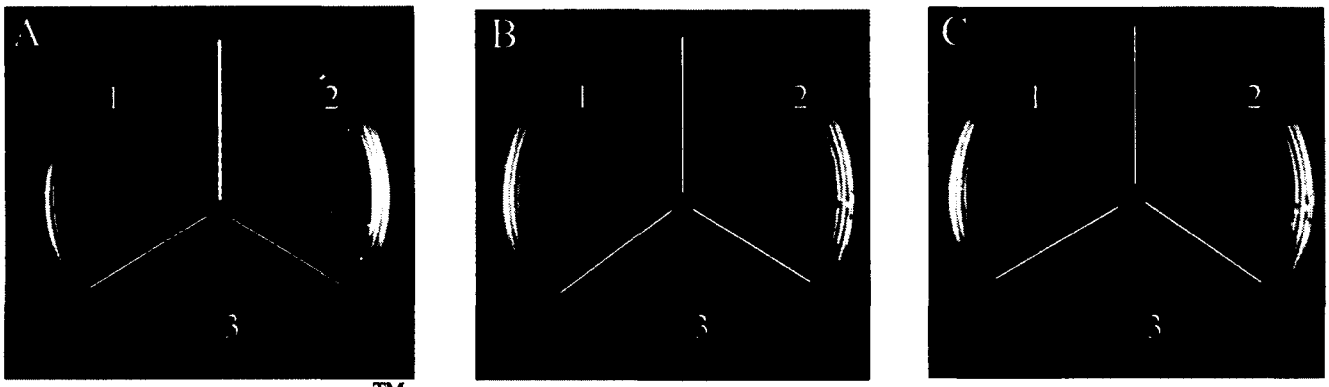

Figure 3. Growth of Origami™ (DE3) cells on LB-Ampicillin plates after transformation with varying dosages of the L104R, E126D and Wild-type APE1 pET15B plasmid constructs. Plates were incubated at 37°C for 24 hours.
A: 100 ng plasmid of 1) pET15b-L104R; 2) pET15B-APE1; 3) pET15b-E126D
B: 300 ng plasmid of 1) pET15b-L104R; 2) pET15B-APE1; 3) pET15b-E126D
C: 500 ng plasmid of 1) pET15b-L104R; 2) pET15B-APE1; 3) pET15b-E126D

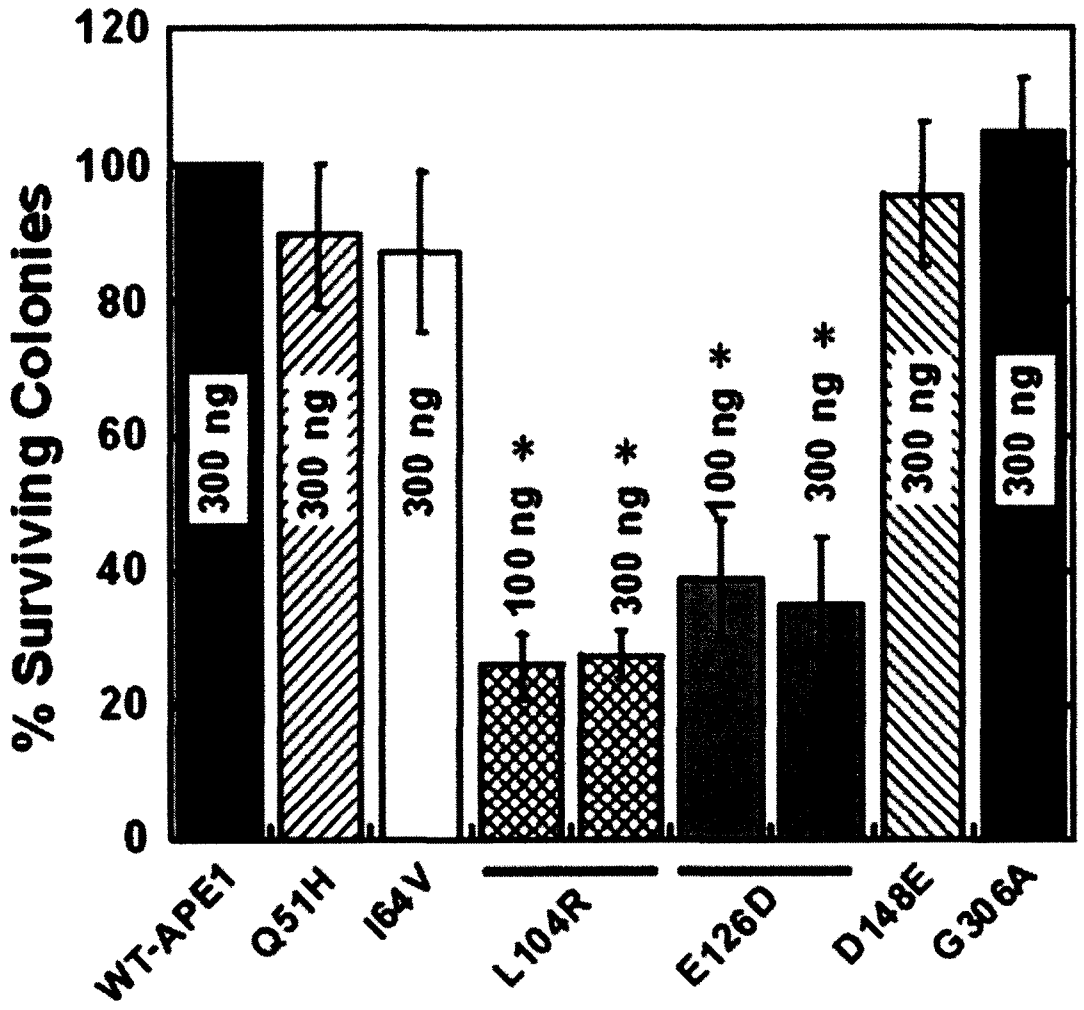

Figure 4. Percent surviving colonies of Origami™ (DE3) cells post transformation with the indicated pET15b plasmid construct and dosage normalized to Wild-type APE1 survival. L104R and E126D at both 100 ng and 300 ng have $P < 0.0001$ using a one way ANOVA test as indicated by *. (n = 3-5)

To further confirm the results obtained using the plate protocol, a second assay was developed using Origami™ (DE3) cells. In contrast to the plate assay, the second broth assay measures doubling time of Origami™ (DE3) cells in liquid culture, where a longer doubling time indicates increased cytotoxicity and a lower doubling time indicates decreased cytotoxicity. Additionally, as an advantage, this method no longer counts surviving colonies and thus eliminates the dependency on transformation efficiency. Initially, wild-type APE1 and empty

vector were transformed. From this, it was determined that wild-type APE1 resulted in a longer doubling time (1.78 +/- 0.17 hours) than the empty vector (1.22 +/- 0.16), indicating cell cytotoxicity due to ribonuclease activity of APE1 (Figure 5 and Table 4). Thus, further experiments with the population variants were carried out. From pooling and analyzing 3 biological replicates, the assay determined that the L104R and E126D APE1 population variants had significantly longer doubling times (2.84 +/- 0.15 and 2.48 +/- 0.19 hours, respectively) as compared to that of the wild-type APE1 or the empty vector (Figure 5 and Table 4). The remainder of the population variants did not show any significant changes in doubling time (Table 4).

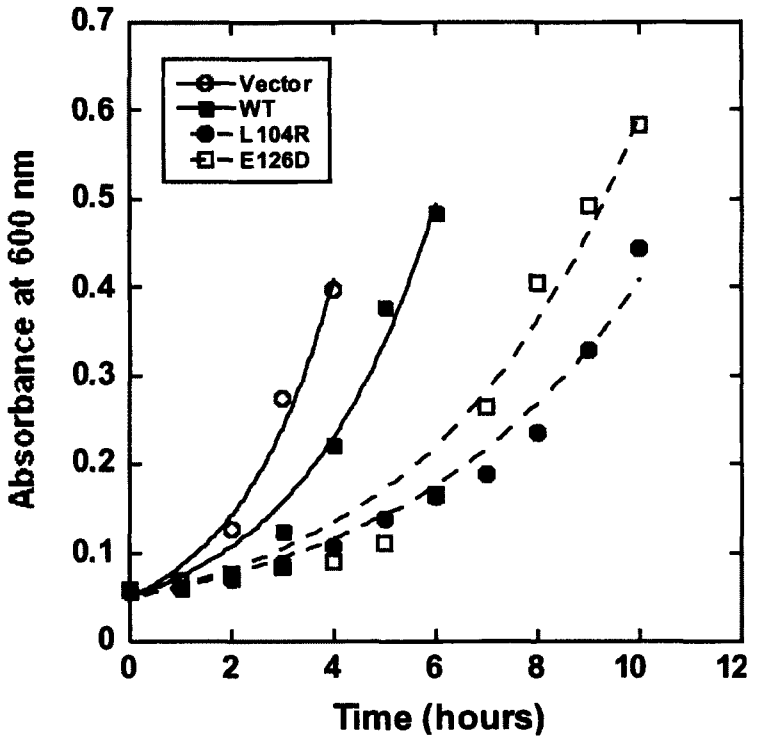

Figure 5. Example growth curve and fitted trend line for determining the doubling time of Origami™ (DE3) cells when transformed with various pET15b plasmid constructs. (n = 1)

Table 4. List of transformation conditions and the resulting doubling times for Origami™ (DE3) cells in the broth protocol. $n = 4$ for empty vector, APE1, L104R and E126D. $n = 3$ for Q51H, I64V, D148E and G306A. Data obtained using KaleidaGraph 4.0

Transformation Condition	Doubling Time (Hours)
pET15b-Empty Vector	1.22 ± 0.16
pET15b-APE1	1.78 ± 0.17
pET15b-Q51H	1.52 ± 0.10
pET15b-I64V	1.58 ± 0.08
pET15b-L104R	2.84 ± 0.15
pET15b-E126D	2.48 ± 0.19
pET15b-D148E	1.63 ± 0.11
pET15b-G306A	1.82 ± 0.08

2.4 Discussion

Origami™ (DE3) cells have been used in many instances for generating properly folded and functional proteins which can then be used for many downstream applications. Some of these include NMR studies (Ponniah *et al.* 2010), immunological studies (Wang *et al.* 2010), and functional studies for screening ribonucleolytic activities of proteins (Raines *et al.* 2006; Raines *et al.* 2008). As such, the proteins expressed in this study are properly folded wild-type APE1 and APE1 population variants. It may not be clear which function or functions of APE1 (ribonuclease, AP-DNA nuclease, redox regulation of transcription factors) are responsible for any observed effects in this experiment. However, given that two of the major other functions of APE1 (AP-DNA nuclease and redox regulation of transcription factors) are based on eukaryotic

specific cellular functions (eukaryotic gene expression regulation and eukaryotic genomic stability regulation) it is unlikely that these functions are responsible for the cytotoxicity observed here in a prokaryotic system. Hence, the observed cytotoxicity is likely attributable to the endoribonuclease activity of APE1.

The results of this study clearly demonstrated the ability of these assays to determine an increase in cellular cytotoxicity. This was observed for wild-type APE1 transformed cells compared to no exogenous protein-expressed cells (empty vector) (Figure 2A, section 2 versus section 1). Similarly, the L104R and E126D-transformed cells demonstrated increased cytotoxicity as compared to wild-type APE1-transformed cells (Figure 4 and Table 4). These results agree with previous experiments where RNase A demonstrated cytotoxicity (Raines *et al.* 2006). Furthermore, subsequent experiments using both the plate and broth protocols in our lab have demonstrated cellular cytotoxicity for a potential ribonuclease, 60S ribonuclease protein L9 (RPL9) (unpublished data).

The Q51H, I64V, D148E, and G306A APE1 variants were found to have no increased cytotoxicity in either assay (Figure 4, Table 4). As such, these variants possess endoribonuclease activity which is comparable to wild-type APE1. However, the D148E and G306A variants were shown to have decreased ribonucleolytic activity compared to wild-type APE1 in previous *in vitro* endoribonuclease assays (Kim *et al.* 2012). The *in vitro* ribonuclease activity of the D148E and G306A APE1 variants should have translated to an increase in surviving colonies in the plate method or a decrease in doubling time in the broth method. However, neither scenario was observed (Figure 2, D148E and G306A versus wild-type APE1 and Table 4, D148E and G306A versus wild-type APE1). It is possible that both these assays are simply not sensitive enough to

detect differences in decreased endoribonuclease activity between APE1 population variant types.

In contrast, the L104R and E126D APE1 variants were shown to be significantly more cytotoxic than wild-type APE1 to the Origami™ (DE3) cells (Figure 4, Table 4), suggesting that these variants have enhanced endoribonuclease activity as compared to wild-type APE1. In the plate protocol, this was observable for transformations at two plasmid concentrations, 100 ng and 300 ng (Figure 4). However, the trend was no longer true at 500 ng (Figure 3C, section 1 versus section 2). This result may be explained by the following: qualitatively, there is an increasing number of surviving colonies as the concentration of plasmid for transformation is increased (Figure 3A versus Figure 3B versus Figure 3C). Such an observation has been observed previously, where an increase in the concentration of pGEX4T3-Angiogenin resulted in an increase in the number of surviving colonies (Raines *et al.* 2006). This may indicate greater transformation efficiency as plasmid concentration is increased and may serve as an explanation for the observed 500 ng plasmid transformation reported here: transformation efficiency may simply be outpacing cytotoxicity at this concentration resulting in a surplus of colonies for all transformation conditions and subsequent masking of cytotoxicity variation. The remaining cytotoxicity of E126D at this concentration (Figure 3C, section 3) may imply a stronger cytotoxicity for this variant as compared to L104R. For the broth assays, the doubling time of L104R and E126D were significantly higher than that of the wild-type APE1 (Section 2.2). Since this is the first study to use Origami™ (DE3) broth assay to assess ribonuclease activity, there are no previous benchmarks to compare the results obtained here. However, unpublished results in our lab for a potential ribonuclease, 60S ribonuclease protein L9 (RPL9), also showed increased doubling times. This, as well as the results from the plate assay, demonstrates an

increase in ribonuclease activity for the L104R and E126D variants as compared to the wild-type APE1.

The observed cytotoxicity of the L104R and E126D variants correlates with altered *in vitro* endoribonuclease activity for these variants. The L104R and E126D variants generated cleavage sites on a *c-myc* CRD RNA substrate that are unique (Kim *et al.* 2012). These variants are also especially active *in vitro*, with RNA cleaving activity observed even in the presence of the powerful RNase inhibitor, RNasin (Kim *et al.* 2012). The results from the plate experiments may provide some insight into the relative ribonucleolytic activity of these variants in cells. Both variants exhibited a 30% to 40% reduction in surviving colonies as compared to the wild-type APE1 (Figure 4). Previous experiments, as well as data obtained in this thesis, have shown that a ribonuclease with strong activity, such as RNase A, will result in complete cellular death with no surviving colonies (Smith *et al.* 2006). The weaker cytotoxicity effect of APE1 demonstrates that APE1 has weak ribonuclease activity when compared to RNase A. However, the increased cytotoxicity by the variants shows that they have increased or altered ribonucleolytic activity as compared to the wild-type APE1. The level of cytoxicity of the variants displayed here do not match that of RNase A, suggesting that these variants do not possess strong ribonucleolytic activity as compared to that of RNase A. In summary, the L104R and E126D variants have stronger ribonucleolytic activity than that of the wild-type APE1 while possessing weaker activity than that of the potent and well-studied RNase A.

The mechanism for the observed increase in cytotoxicity for L104R and E126D may be explained by the molecular changes that these mutations cause to the APE1 protein. A study using molecular modeling has previously demonstrated that both the L104R and E126D variants may be of importance to the global conformation of the APE1 protein. Amino acid 104 of APE1

is positioned in a loop between β–sheet number 2 and α–helix number 3 (Hadi *et al.* 2000). This area is directly adjacent to the nucleic acid binding and recognition site. It was hypothesized that, upon substitution of the lysine residue with an arginine residue, the hydrophobic interactions between L104 and its neighboring residues (L72, L108, and W119) is disrupted resulting in a change in the structure of APE1 (Hadi *et al.* 2000). This would result in a novel positively charged region of the protein which may translate into an increased attraction of the negatively charged RNA substrate into the binding and catalysis pocket. This could explain the altered *in vitro* RNA cleaving activity previously observed (Kim *et al.* 2012) as well as the possible increase in RNA cleaving activity observed in this experiment for this variant (Figure 4, Table 4). E126D presents an interesting case as the amino acid substitution here results in simply a reduction of one methylene group and no modifications to the overall or local charge of the protein. However, location of this residue is also positioned in a vital location relative to the secondary structure of APE1. E126 is located between β–sheet number 3 and 4 and is in close proximity to the DNA phosphate backbone (Hadi *et al.* 2000). This shortening of the amino acid side-chain may result in a change to the mobility of the β–sheets resulting in an increased opportunity for RNA binding and cleavage. Future investigations using crystal structures of these variants bound to RNA substrates could elucidate the exact binding and cleaving mechanisms which lead to this observed cytotoxicity.

To summarize, the population variants L104R and E126D had increased cytotoxicity to Origami™ (DE3) cells in both assays used, suggesting an increase in ribonuclease activity. The remaining population variants, including D148E, demonstrated no discernible difference in cytotoxicity as compared to wild-type APE1, suggesting no change in ribonuclease activity.

How biologically relevant are these increases in cytotoxicity for the L104R and E126D variants? Previously, it has been demonstrated that the angiogenesis function of angiogenin is caused by the ribonucleolytic activity of the protein (Kelemen *et al.* 1999). This function is vital for angiogenesis in tumor formation. In addition, the ribonucleolytic function of angiogenin was clearly demonstrated by death of these Origami™ (DE3) cells (Smith *et al.* 2008). As such, any observed changes to the survivability of Origami™ (DE3) cells due to the population variants of APE1 are likely to translate into a biologically relevant eukaryotic system.

Chapter 3

Eukaryotic Systems in Assessing the Effects of Over-expressing APE1 Population Variants

3.1 Introduction

This chapter describes the investigations into the possible phenotypic changes in eukaryotic cells upon over-expression of the two APE1 population variants, L104R and E126D. Preliminary investigations into the possible molecular mechanisms for the observed phenotypic changes were undertaken using microarray analysis and quantitative real time polymerase chain reaction (qRT-PCR).

In Chapter 2, both the L104R and E126D variants were shown to possess enhanced cytotoxicity in a prokaryotic system. This correlated with previous *in vitro* work which demonstrated unique RNA-cleaving activity of these variants (Kim *et al.* 2012). Various APE1 population variants have previously been identified to be associated with cancers (Narter *et al.* 2008; Pardini *et al.* 2008; Kasahara *et al.* 2008; Jiao *et al.* 2006; Tse *et al.* 2008; Canbay *et al.* 2011). Therefore, it is warranted to investigate the effect these variants may have upon over-expression in eukaryotic cells. In this thesis, two cancer cell lines were used: human cervical cancer cells (HeLa cells) and human hepatoma cancer cells (HepG2). Initial experiments were performed to determine if endogenous APE1 plays a role in the growth of HeLa and HepG2 cells. To achieve this, small interfering RNA (siRNA) technology was used to knock down endogenous APE1 expression. Growth analyses, as measured by an increase in the number of cells, were carried out using 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide

(MTT) Assays. Concurrent research in Dr. Lee's lab had suggested that localization of APE1 to P-bodies, a focal point of mRNA turnover, was facilitated by stress. To induce such stress, sodium arsenite was used in the HepG2 growth analyses to facilitate localization of APE1 and its population variants to the P-bodies.

Gene expression analysis studies were conducted in an effort to link altered gene expression, through the endoribonuclease activity of the APE1 variants, with the displayed altered phenotype when the variants were over-expressed in HepG2 cells. Two methodologies were used: 1) Microarray analysis, to quickly screen a large number of potential mRNA targets of the population variants; and 2) qRT-PCR, to confirm the results obtained from the microarray analyses.

3.2 Methodology

3.2.1 Preparation of pCMV5.1-APE1-FLAG Constructs

The pCMV5.1-FLAG vector was purchased from OriGene Technologies Inc. (Rockville, MD) containing the APE1 cDNA sequence. This construct was subsequently used to generate pCMV5.1-L104R-FLAG and pCMV5.1-E126D-FLAG constructs *via* site-directed mutagenesis as previously described in section 2.1.1. Table 5 lists the primers used for the site-directed mutagenesis. Sequence identity was confirmed by DNA sequencing performed by Macrogen Inc. (Seoul, South Korea).

Table 5. Sequence identity of primers used for generation of population variant pCMV5.1-FLAG plasmid constructs. Highlighted nucleotides are responsible for the amino acid substitution.

Plasmid Construct	Forward Primer	Reverse Primer
L104R	5'-TCA GAG AAC AAA CGA CCA GCT GAA CTT-3',	5'-AAG TTC AGC TGG TCG TTT GTT CTC TGA-3'
E126D	5'-CCT TCG GAC AAG GAC GGG TAC AGT GGC-3'	5'-GCC ACT GTA CCC GTC CTT GTC CGA AGG-3'

3.2.2 Growth Rates of HeLa cells with APE1 Knockdown

Human cervical cancer cells (HeLa) were purchased from the American Type Culture Collection (ATCC) and were subsequently cultured in T25 flasks containing Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. The HeLa cells were then trypsinized for 5 minutes and the cells expanded using 10 ml of fresh medium. Cell counts were then determined using a Bright-Line Haemocytometer (Hausser Scientific) and the cell density adjusted to appropriate levels for plating cells at a concentration of $5x10^4$ cells per well using MEM. Cells were subsequently plated into 6 well tissue culture plates, in experimental duplicates, at $5x10^4$ cells per well in a total of 2 ml of volume. The next day, cells were transfected with either siRNA targeting APE1 or a scramble negative construct *via* Lipofectamine 2000 (Invitrogen, United States) using the following protocol: mixing of 2.5 µl of siRNA (final concentration of 20 nM) or scramble negative DNA (final concentration 20 nM) with 250 µl Opti-MEM and incubation for 15 minutes; mixing of 2.5 µl of Lipofectamine 2000 with 250 µl Opti-MEM and incubation for 15 minutes; mixing of these two Opti-MEM fractions and incubation for 30 minutes; replacement of media with 1.5 ml of fresh MEM medium; finally, addition of 500 µl of combined siRNA or scramble negative and Lipofectamine to the wells of the 6 well plates containing cells. The transfected cells were incubated overnight at 37°C in 5%

CO_2. The sequences of the double stranded RNA sequences used in this experiment are in Table 6.

Table 6. Sequence identity of double stranded RNA used for transfection into HeLa and HepG2 cells. The majority of the constructs are comprised of ribonucleotides (r). The sense constructs contain two deoxyribonucleotides (d).

dsRNA Construct	Sense	Antisense
APE1-sRNAi	r(GUCUGGUACGACUGGAGUACCGG)dCA	r(UGCCGGUACUCCAGUCGUACCAGACCU)
Scramble Negative-dsRNAi	r(CUUCCUCUCUUUCUCUCCCUUGU)dGA	r(UCACAAGGGAGAGAAAGAGAGGAAGGA)

Cells were trypsinized and counted, then expanded with fresh medium and re-plated, in triplicates, into 96-well tissue culture plates in concentrations of 500, 1000, 1500 or 2000 cells per well in a total volume of 200 µl each. The cells were incubated overnight at 37°C in 5% CO_2.

Cells were then subjected to MTT assay analysis for the subsequent 5 days in 24 hour intervals – 24, 48, 72, 96 and 120 hours after plating. A solution containing the MTT dye Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich, United States) was diluted to $1/5^{th}$ concentration using fresh MEM medium. Subsequently, 50 µl of this solution was added to the wells of the 96 well plates and left to incubate at 37°C and 5% CO_2 for 3 hours. Following incubation, 220 µl of medium was aspirated from each well and replaced with 150 µl of DMSO to dissolve and suspend the dye. Absorbance at 570 nm was then measured on a spectrophotometer as a measure of cell growth. Growth curves were prepared using Kaleidagraph 4.0™ (Synergy Software, USA). A total of 3 biological replicates were performed.

3.2.3 Growth Rates of HeLa Cells Over-expressing APE1 Population Variants

HeLa cells were prepared for transfection in 6-well tissue culture plates as previously described in section 3.2.2. The transfection mixture for these experiments consisted of 1 μg of each of pCMV5.1-FLAG, pCMV5.1-Wild-typeAPE1-FLAG, pCMV5.1-L104R-FLAG, or pCMV5.1-E126D-FLAG plasmid constructs. Subsequent transfection, incubation and MTT analysis were performed as previously described in section 3.1.2. Growth curves were produced using Kaleidagraph 4.0™ (Synergy Software, USA) and a total of 3 biological replicates were performed.

3.2.4 Preparation of pCMV6-XL5-APE1 Constructs

pCMV6-XL5-APE1 constructs were prepared by subcloning the APE1 or population variant (L104R and E126D) cDNA sequences from the pCMV5.1-Wild-typeAPE1-FLAG plasmid constructs used in the previous HeLa experiments. The wild-type APE1, L104R and E126D cDNA sequences were amplified *via* PCR to remove the FLAG tag and 3' UTR using the following primers: forward: 5'-CGA GGT CTG GTC CGT CTG GAG TAC-3 and reverse: 5'-GTA CTC CAG ACG GAC CAG ACC TCG-3. Amplification of the cDNA was carried out in a MJ Research MiniCycler with the following PCR cycle: Denaturation at 95°C for 45 seconds, primer annealing at 55°C for 45 seconds and extension at 68°C for 10 minutes for a total of 18 cycles. pCMV6-XL5 was then purchased from OriGene Technologies Inc. (Rockville, MD).

Approximately 1 μg of each type APE1 cDNA, generated from the PCR reactions (Wild-type, L104R and E126D), and 1 μg of pCMV6-XL5 empty vector were then subjected to an overnight double restriction digest using EcoRI (Invitrogen, United States) and XbaI (New England BioLabs, Massachusetts). The digestion was carried out at 37°C using 10 units each of

enzyme in a total reaction volume of 100 µl. Ligation reactions were then carried out using an insert to vector molar ratio of 3:1. The restriction digested APE1 cDNA inserts, restriction digested pCMV6-XL5 vector and 1 unit of T4 DNA Ligase (New England Biolabs, Massachusetts) were incubated for 2 hours at room temperature to generate ligated pCMV6-XL5-APE1 constructs. Identification of the clones were carried out by restriction digest and comparison of subsequent insert band sizes compared to previously established APE1 cDNA sequence size (obtained from the pCMV5.1-FLAG constructs) on a 1% agarose gel. Identities of the plasmid constructs were confirmed by DNA sequencing at Macrogen Inc. (Seoul, Sorth Korea).

3.2.5 Confirmation of Knockdown of APE1 in HepG2 Cells

Human hepatoma cancer cells (HepG2) were purchased from ATCC and subsequently maintained and passaged in T25 flasks containing MEM medium supplemented with 10% fetal bovine serum at 37°C at 5% CO_2. Cells were counted and plated into 6-well tissue culture plates, in experimental duplicates, at $5x10^4$ cells per well in a total of 2 ml of volume. The next day, cells were transfected with either siRNA targeting APE1 or scramble negative constructs *via* Lipofectamine 2000 (Invitrogen, United States) as previously described in section 3.2.2. Table 6 describes the double stranded RNA sequences used for this experiment. The transfected cells were incubated overnight at 37°C in 5% CO_2.

At 48 and 72 hours post transfection, total cellular lysates were collected from the siRNA and scramble negative construct transfected wells. From the cellular lysates, 200 µl of 8M Urea was added to each well and the cells scraped from the wells using a plate scraper. The lysates were then subjected to 3 cycles of freezing at -80°C, thawing at 42°C and vigorous vortexing.

Samples were centrifuged at 13000 RPM in an Eppendorf Centrifuge 5415 R for 10 minutes at 4°C. The supernatants containing the cellular proteins were collected and stored at -20°C until further use. Protein concentration was determined using a standard Bradford assay (Bio-Rad, California).

From the total cellular lysates, 80 μg of protein for each sample type were loaded onto a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and electrophoresed for 1 hour at 100V. Upon completion of electrophoresis, proteins were transferred from the SDS polyacrylamide gel onto Amersham™ Hybond™-ECL paper (GE Healthcare, United Kingdom) *via* a Bio-Rad transfer device at 100V for one hour. The ECL paper was then subjected to Western analysis *via* incubation with 1/3000 dilution human APE1 and human β-Actin monoclonal antibodies (Affinity Bioreagents, Colorado) for 2 hours at room temperature and 1/3000 horseradish peroxidase conjugated secondary mouse monoclonal antibodies (Promega, Wisconsin) for one hour at room temperature. Chemiluminescence of samples were achieved using Pierce SuperSignal© West Pico Chemiluminescent Substrate kit and the resulting images were captured using an Alpha Innotech ChemiImager cabinet and FluroChem 5500 software.

3.2.6 Growth Rates of HepG2 Cells with APE1 Knockdown

HepG2 cells were prepared and transfected with siRNA and scramble negative sequences (Table 4) as previously described in secion 3.1.5. Cells were then counted and plated to 96 well plates in experimental triplicates. Finally, water or sodium arsenite (final concentrations of 0 mM, 0.5 mM or 1.0 mM of sodium arsenite) (Sigma-Aldrich, United States) were added to the wells containing the cells.

MTT analysis for 0, 24, 48, 72, 96, 120 and 144 hours post-transfection were carried out as previously described in section 3.1.2. The resulting data was used to generate growth curves in Kaleidagraph 4.0™ (Synergy Software, USA). A total of 3 biological replicates were performed.

3.2.7 Confirmation of Over-expression of APE1 and its Variants in HepG2 Cells

HepG2 cells were prepared as described in section 3.1.5 and transfected with 1 ug each of the pCMV6-XL5-Vector, pCMV6-XL5-Wild-typeAPE1, or pCMV6-XL5-L104R plasmid constructs. At 72 hours post transfection, total cellular protein were collected from the pCMV6-XL5-Vector, pCMV6-XL5-Wild-typeAPE1, or pCMV6-XL5-L104R transfected wells using the standard Urea lysis protocol as described previously in section 3.1.5.

Total cellular protein was loaded directly onto Amersham™ Hybond™-ECL paper. Protein loaded per dot ranged from 25 μg to 1.56 μg in 2 fold dilution steps. In addition, 0.1 μg of recombinant human APE1 was loaded as a control. The ECL paper containing the protein samples were incubated with human APE1 monoclonal antibody (Affinity Bioreagents, Colorado) (1/3000 dilution) or human β-actin monoclonal antibody (Sigma-Aldrich, United States) (1/3000 dilution) for 2 hours at room temperature. The ECL paper was then incubated 1/3000 dilution horseradish peroxidase conjugated secondary monoclonal mouse antibodies (Promega, Wisconsin) for 1 hour at room temperature. Chemiluminescence of samples were achieved using Pierce SuperSignal© West Pico Chemiluminescent Substrate kit and the resulting images were captured using an Alpha Innotech ChemiImager cabinet and FluroChem 5500 software.

3.2.8 Growth Rates of HepG2 Cells Over-expressing APE1 Population Variants

HepG2 cells were prepared as described in section 3.1.5 and transfected with 1 μg each of the pCMV6-XL5-Vector, pCMV6-XL5-Wild-typeAPE1, pCMV6-XL5-L104R, or pCMV6-XL5-E126D plasmid constructs. Cells were counted and plated to 96 well plates in experimental triplicates. Finally, water or sodium arsenite (final concentrations of 0 mM, 0.5 mM or 1.0 mM sodium arsenite) (Sigma-Aldrich, United states) were added to the wells containing the cells.

MTT analysis for 0, 24, 48, 72, 96, 120 and 144 hours post transfection were carried out as previously described in section 3.2.2 and the resulting data were used to generate growth curves in Kaleidagraph 4.0™ (Synergy Software, USA). A total of 3 biological replicates were performed. Subsequent two way ANOVA statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, California).

3.2.9 Isolation of mRNA for Microarray and qRT-PCR Analysis of Gene Expression

HepG2 cells were prepared and transfected as previously described in section 3.2.2 with 1 μg each of the pCMV6-XL5-Vector, pCMV6-XL5-Wild-typeAPE1, or pCMV6-XL5-L104R vectors. Forty-eight hours after transfection, mRNA was harvested from each transfection type using the *mir*Vana™ mRNA isolation kit (Ambion, Texas). To each transfection type, 200 μl of Lysis/Binding buffer were added and the cells mechanically scraped from the wells using a pipet tip. Homogenate Additive was added to a $1/10^{th}$ volume of the lysis mixtures and the mixtures were kept on ice for 10 minutes. An equal volume of acid phenol chloroform (pH = 4.5) was then added to the mixtures and the mixtures were centrifuged at 5 minutes at 10,000RPM. The upper phase containing nucleic acids were carefully aspirated and transferred to the supplied filter cartridges. The cartridges containing mRNA were then washed once with 700 μl of Wash Buffer

1 and twice with 500 µl of Wash Buffer 2. mRNA was eluted from the collection cartridges using 90°C nuclease free water. mRNA was stored at -80°C until further use. RNA samples were then subjected to DNA cleanup *via* using 1000 units of DNase I (Promega, Wisconsin) incubated at 1 hour at 37°C. mRNA samples were then measured using a Nanodrop spectrophotometer to determine the A_{260}/A_{280} OD ratio and aliquoted into 20 µl aliquots for storage at -80°C until further use.

3.2.10 Microarray Analysis and Preliminary qRT-PCR

mRNA harvested from a single biological replicate of pCMV6-XL5- Vector, pCMV6-XL5-Wild-typeAPE1, and pCMV6-XL5-L104R transfected HepG2 cells were sent to the Hospital for Sick Children in Toronto for microarray analysis. Two genes of interest were chosen for subsequent qRT-PCR analysis – BCL2A1 and RASEF. Primers used for the qRT-PCR experiments were chosen based on previous gene expression studies analyzing these genes – BCL2A1 from a study concerning anti-apoptosis in human neutrophils (Lee *et al.* 2006) and RASEF from a paper identifying RASEF as a tumour suppressor gene in uveal melanoma (Maat *et al.* 2008). c-MYC and β-Actin primers were chosen from gene study experiments previously undertaken in Dr. Lee's lab (Barnes *et al.* 2009). Table 7 lists the gene identities and subsequent primers used for the qRT-PCR analysis. In addition to the mRNA harvested for the microarray analysis, one additional biological replicate of mRNA was used for the qPCR experiments.

Table 7. List of primers and sequence identity of primers used for qRT-PCR analysis

Gene Identity	Forward Primer	Reverse Primer
BCL2A1	5'-CCA GGC AGA AGA TGA CAG-3'	5'-CTG GCA GTG TCT ACG GAC-3'
RASEF	5'-TTC CTC TTC CAA CTC ACT CAA CTG-3'	5'-ATC AGA CTT CAA AGC ACA GAA ATG-3'
c-MYC	5'-ACG AAA CTT TGC CCA TAG CA-3'	5'-GCA AGG AGA GCC TTT CAG AG-3'
β-Actin	5'-TTG CCG ACA GGA TGC AGA AGG A-3'	5'-AGG TGG ACA GCC AGG CCA GGA T-3'

First strand cDNA was synthesized for all subsequent qRT-PCR applications using iScript cDNA™ Synthesis Kit (Bio-Rad). From the total RNA, 1 μg of RNA from each transfection type was mixed with 4 μl of 5X reaction mix, 1 μl of reverse transcriptase and nuclease-free water to a final volume of 20 μl. First strand cDNA synthesis was carried out in a MJ Research MiniCycler with the following PCR cycle: 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes and storage at 4°C until removal from the MiniCycler unit. cDNA was synthesized fresh for each experiment and was used immediately in downstream applications upon synthesis. All subsequent qRT-PCR applications were carried out using an IQ5 qRT-PCR cycler (Bio-Rad, California). The dye used for detection of amplification was iQ™ SYBR® Green Supermix (Bio-Rad, California).

Melt curve analyses were carried out on two primers sets (BCL2A1 and RASEF). cDNA was pooled from three transfection conditions (pCMV6-XL5-Vector, pCMV6-XL5-Wild-typeAPE1 and pCMV-XL5-L104R) and the analyses conducted at a range of temperatures from 52°C to 62°C. Melt curves were generated using the iQ5 software (Bio-Rad, California). Gene expression studies were carried out using the following cycle conditions: 3 minutes at 95°C for one cycle followed by 40 cycles of 10 seconds at 95°C and 30 seconds at 60°C. Gene expression

values were calculated by the iQ5 software (Bio-Rad, California) using the ddCT method with β-Actin acting as the reference gene as per previously established in the Lee lab (Barnes *et al.* 2009).

3.3 Results

3.3.1 Growth Rates of HeLa cells with APE1 Knockdown

The first step towards analyzing the effects of APE1 population variants on mammalian cells was to determine if APE1 plays a role in the modulation of growth rate in HeLa cells. To determine this, experiments were performed to knock down endogenous levels of APE1 protein and observe the effects upon growth rate. Four cell densities, based on previous MTT experiments carried out in Dr. Lee's lab using mammalian cells (Bassett 2007), were chosen for this experiment – 500, 1000, 1500 and 2000 cells per well. Qualitative observations from a single biological replicate were analyzed for each cell density. Over a five day period, there was no observable difference in growth rates between knockdown APE1 HeLa cells versus control HeLa cells at the cell densities of 500 cells per well (Figure 6A), 1000 cells per well (Figure 6B), 1500 cells per well (Figure 6C) or 2000 cells per well (Figure 6D) over the 5 day period. Two additional biological replicates were performed, with similar qualitative growth patterns observed (data not shown).

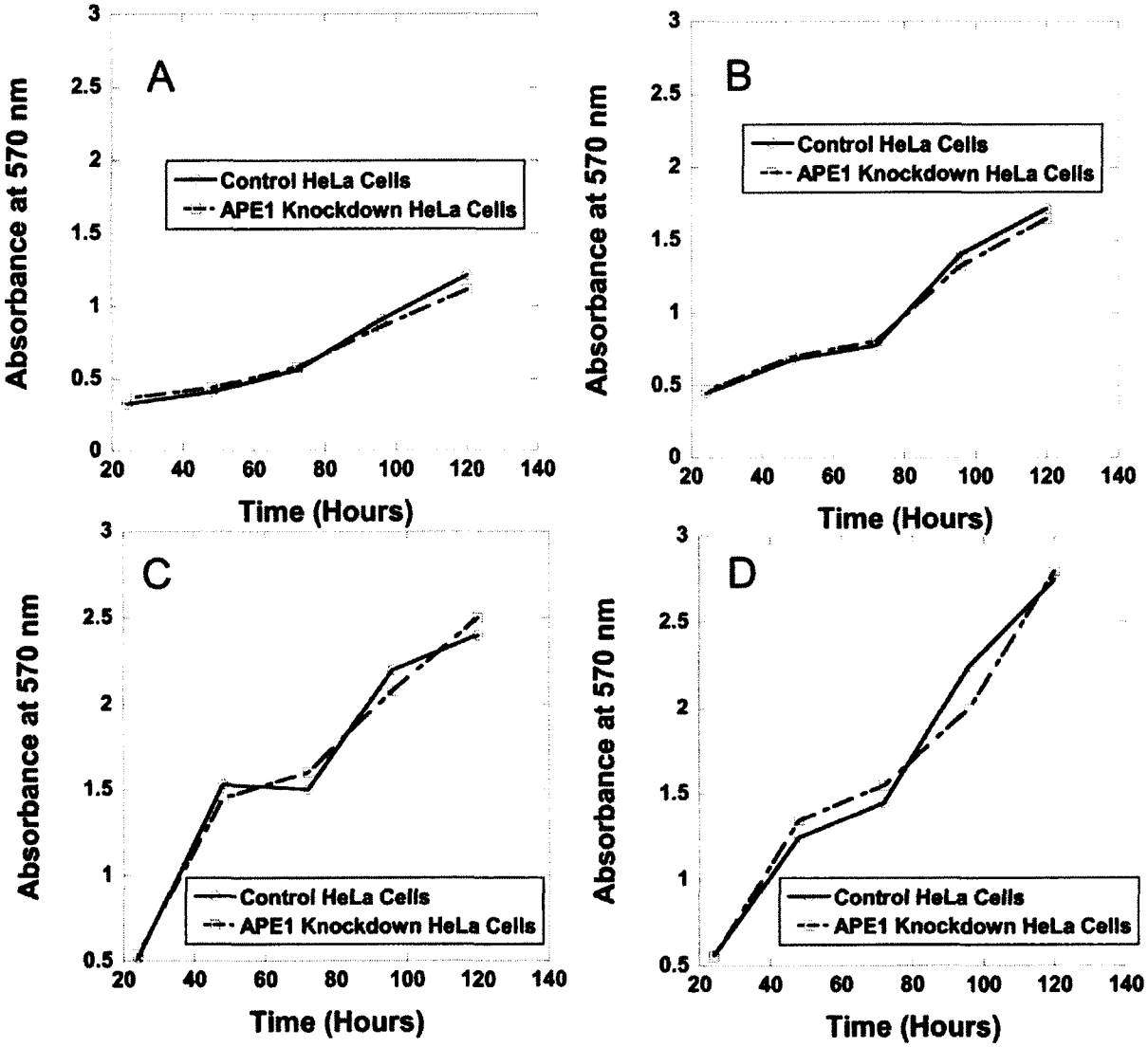

Figure 6. Representative growth rate of HeLa cells over a five day period when transfected with either siRNA targeting APE1 (APE1 Knockdown HeLa cells) or scramble negative RNA (Control HeLa cells). A single biological replicate is shown for each cell plating density ($n = 1$). All biological replicates growth displayed this growth pattern.
A: 500 cells per well; B: 1000 cells per well; C: 1500 cells per well; D: 2000 cells per well

3.3.2 Growth Rates of HeLa Cells Over-expressing APE1 Population Variants

The next experiment was to over-express the L104R and E126D APE1 variants in HeLa cells to observe potential changes in growth rate as compared to the wild-type APE1. The same four cell densities used in section 3.2.1 were chosen for these experiments – 500, 1000, 1500 and 2000 cells per well. Qualitative observations from a single biological replicate were analyzed for each cell density. There were no observable differences between growth rates between L104R APE1 HeLa cells or E126D APE1 HeLa cells versus wild-type APE1 HeLa cells at the cell densities of 500 cells per well (Figure 7A), 1000 cells per well (Figure 7B), 1500 cells per well (Figure 7C) or 2000 cells per well (Figure 7D) over the 5 day period. In addition, there was no difference in growth rate when comparing wild-type APE1 HeLa cells to that of empty-vector HeLa cells for all cell densities (Figure 7A, 7B, 7C and 7D) at all points during the growth assay. Two additional biological replicates were performed, with similar qualitative growth patterns observed (data not shown).

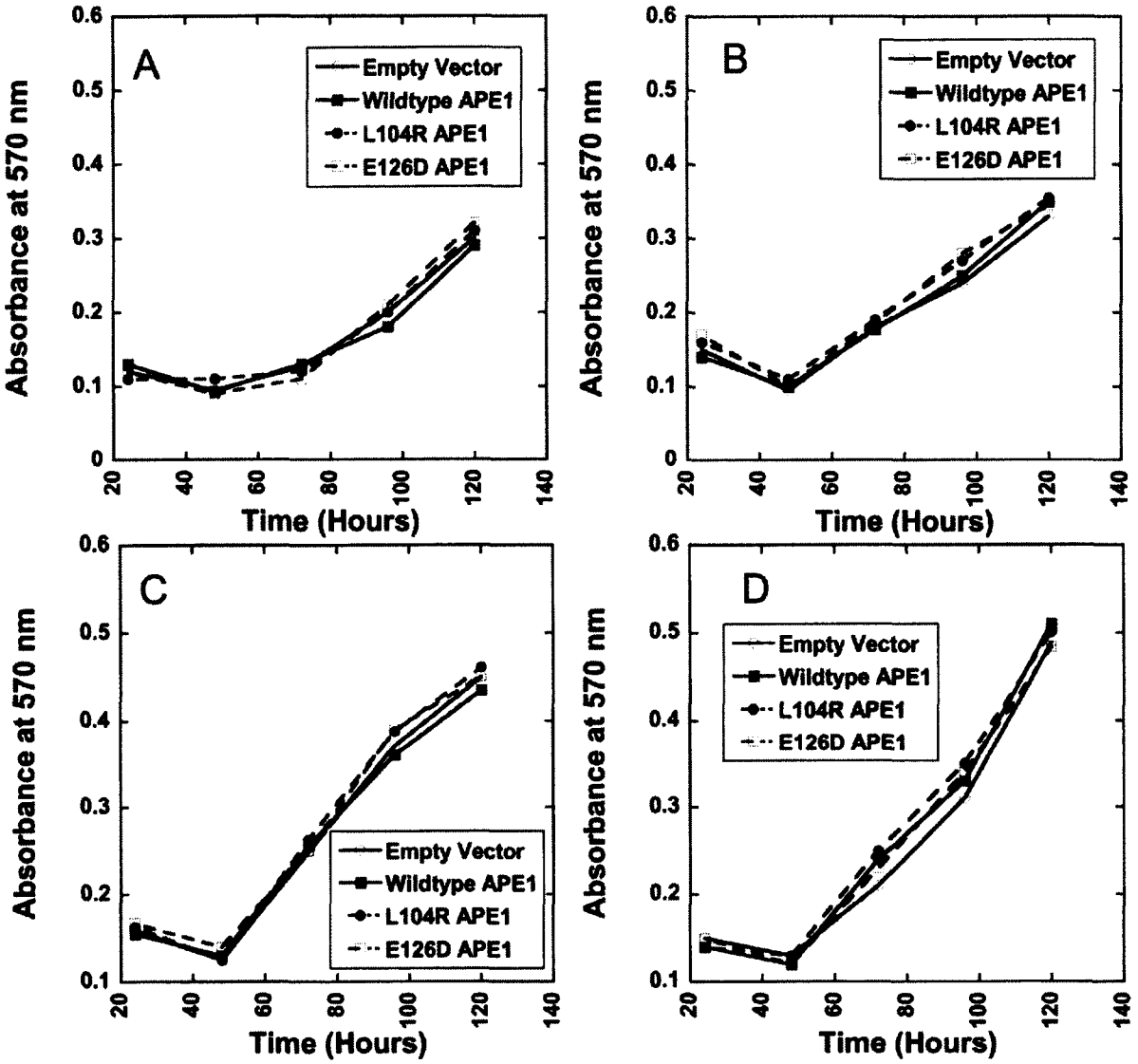

Figure 7. Representative growth rate of HeLa cells over a five day period when transfected with pCMV5-Vector (Empty Vector), pCMV5.1-Wild-typeAPE1-FLAG (Wild-type APE1), pCMV5.1-L104R-FLAG (L104R APE1) or pCMV5.1-E126D-FLAG (E126D APE1). A single biological replicate is shown for each cell plating density (n = 1). All biological replicates displayed this growth pattern.
A: 500 cells per well; B: 1000 cells per well; C: 1500 cells per well; D: 2000 cells per well

3.3.3 Growth Rates of HepG2 Cells with APE1 Knockdown

To assess if APE1 plays a role in the modulation of growth rate in HepG2 cells, APE1 was knocked down and growth rates were observed. A single cell density was chosen for these experiments (2000 cells per well) as the previous HeLa cell experiments demonstrated that cells nearly reached confluency over a 5 day period at this concentration. As HepG2 cells are known to grow slower than HeLa cells, the experiment was expanded to cover six days instead of five days. Concurrent research in Dr. Lee's lab had shown that APE1 localized to the cytoplasm of HepG2 cells upon treatment with 0.1 to 1.0 mM sodium arsenite (Kim S-E, unpublished observation). Thus, three concentrations of sodium arsenite were chosen for this experiment – 0 mM, 0.5 mM and 1.0 mM. Qualitative analysis from a single biological replicate was performed. At 0 mM sodium arsenite, there was no observable change in growth rate between APE1 knockdown HepG2 cells versus control HepG2 cells for the first 24 and 48 hours (Figure 8A). However, starting at 72 hours and continuing on through 144 hours, there was an observable decrease in cell growth rate for APE1 knockdown HepG2 cells as compared to the control HepG2 cells (Figure 8A). At 0.5 mM sodium arsenite, there was no change in growth rate for the first 24 hours; however, there was a noticeable decrease in growth rate for APE1 knockdown HepG2 cells starting at 48 hours continuing through 144 hours (Figure 8B). A similar growth pattern was observed for 1.0 mM sodium arsenite – at 24 hours there was no discernible difference in growth rate with a noticeable decrease in growth rate for APE1 knockdown HepG2 cells starting at 48 hours (Figure 8C). The general effect of the sodium arsenite stressor was cytotoxicity to the HepG2 cells (Figure 8, compare A to B to C) while retaining the overall growth pattern. The highest growth of the HepG2 cells was reduced from a final absorbance (570 nm) value of approximately 2.00 for no sodium arsenite (Figure 8A) to 0.55 for 0.5 mM sodium

arsenite (Figure 8B) to 0.50 for 1.0 mM sodium arsenite (Figure 8C). Two additional biological replicates were conducted and showed the similar qualitative results (data not shown).

To confirm that the change in growth rate observed was due to knockdown of APE1 protein levels, Western blot analysis was performed on proteins isolated from APE1 knockdown HepG2 cells and control HepG2 cells. For 48 hours post transfection, there was a decrease in APE1 protein expression for the APE1 knockdown HepG2 cells as compared to the control HepG2 cells (Figure 9, compare lanes 1 to 2). APE1 protein levels were also reduced at 72 hours post transfection in APE1 knockdown HepG2 cells versus control HepG2 cells (Figure 9, compare lanes 3 to 4). β-Actin levels remain constant throughout both transfection conditions and time (Figure 9, compare lanes 1 through 4) indicating that the knock down of APE1 was specific. This also indicated equal loading of protein samples in each well. The timing of the knock down of APE1 correlates with growth curve data – the 0 mM sodium arsenite growth curves show a decrease in cell growth for APE1 knockdown HepG2 cells starting at 72 hours post transfection (Figure 8A) while the 0.5 mM and 1.0 mM sodium arsenite show decrease of growth starting at 48 hours post transfection (Figure 8B, 8C). These results of suggest that endogenous APE1 plays a role in facilitating the growth of HepG2 cells.

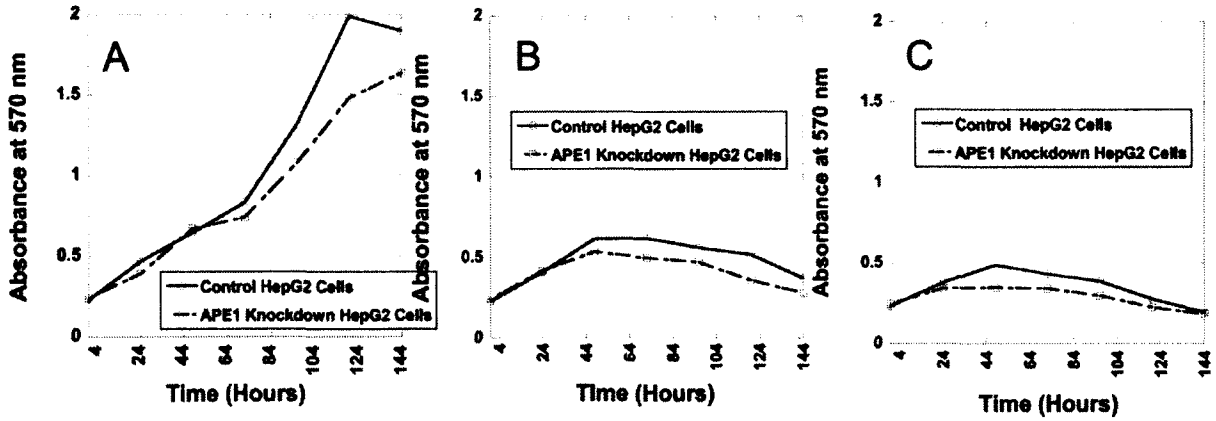

Figure 8. Representative growth rates generated from incubating HepG2 cells transfected with either siRNA targeting APE1 (APE1 Knockdown HepG2 cells) or scramble negative RNA (control HepG2 cells) over a six day period at indicated concentrations of sodium arsenite. A single biological replicate is shown for each concentration of sodium arsenite (n = 1). All biological replicates displayed this growth pattern.
A: 0 mM sodium arsenite; B:0.5 mM sodium arsenite; C: 1.0 mM sodium arsenite

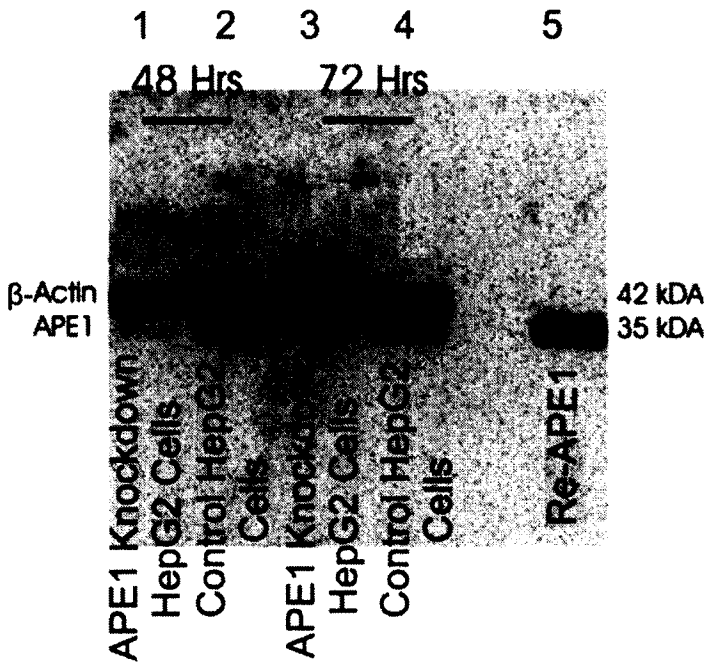

Figure 9.Western blot analysis of protein isolated from HepG2 cells transfected with siRNA targeting APE1 (APE1 Knockdown HepG2 Cells) or scramble negative RNA (Control HepG2 cells). 80 μg total cellular protein was loaded for wells 1-4; 0.1 μg recombinant human APE1 was loaded to lane 5 as a positive control. Samples were subjected to incubation with human APE1 and human β-actin monoclonal antibodies (n = 1).

3.3.4 Growth Rates of HepG2 Cells Over-expressing APE1 Population Variants

We next examined the effect of over-expressing L104R and E126D APE1 variants on the growth of HepG2 cells in the presence or absence of sodium arsenite (0 mM, 0.5 mM and 1.0 mM sodium arnsenite). Qualitative observations were taken from a single biological replicate. At 0 mM sodium arsenite, there was an observable increase in cellular growth rate of wild-type APE1 HepG2 cells as compared to that of empty vector HepG2 cells starting at 72 hours and continuing on throughout the 144 hour test period (Figure 10A). There was also a modest, qualitative increase in cellular growth rates with L104R APE1 HepG2 cells and E126D APE1 HepG2 cells when compared to both wild-type APE1 HepG2 cells and empty vector HepG2 cells at all time periods starting at 24 hours (Figure 10A). A qualitative trend was observed for cells incubated with 0.5 mM sodium arsenite: wild-type APE1 HepG2 cells exhibited enhanced cell growth rate as compared to empty vector HepG2 cells starting at 72 hours while the L104R APE1 HepG2 cells and E126D APE1 HepG2 cells exhibited enhanced growth rate compared to both wild-type APE1 HepG2 cells and empty vector HepG2 cells starting at 72 hours (Figure 10B). However, this trend did not continue with 1.0 mM sodium arsenite incubation; there may have been minor enhanced cellular growth for E126D APE1 HepG2 cells at 48 hours and L104R and E126D HepG2 cells at 96 hours (Figure 10C). This difference is qualitatively reduced compared to that observed in both the 0 mM and 0.5 mM sodium arsenite treated cells. There is no qualitative difference in growth rate between transfection conditions for the remainder of the times analyzed in the 1.0 mM transfection (Figure 10C). Two additional biological replicates were conducted and similar qualitative results were obtained (data not shown).

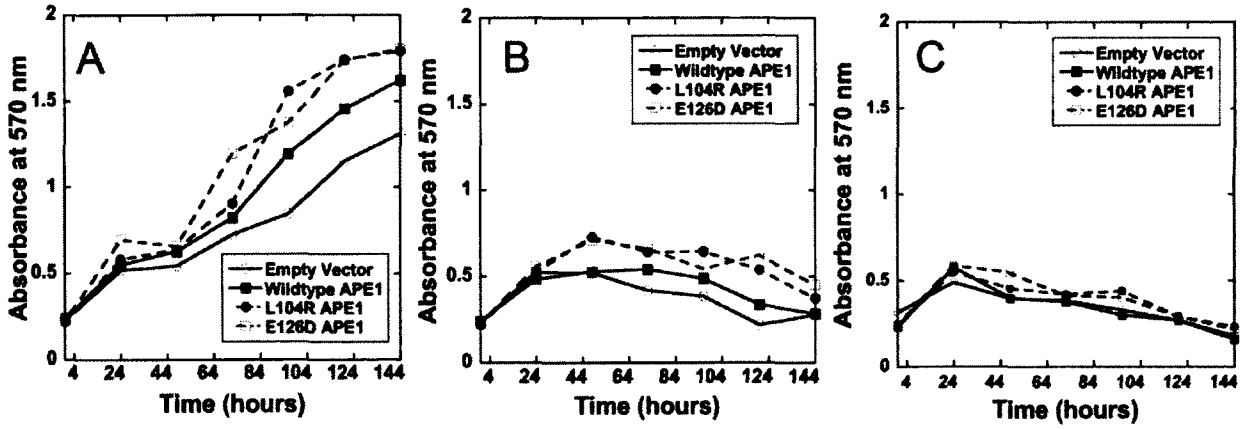

Figure 10. Representative growth curves generated from incubating HepG2 cells transfected with pCMV6-XL5-Vector (Empty Vector), pCMV6-XL5-Wild-typeAPE1 (Wild-type APE1), pCMV6-XL5-L104R (L104R APE1) or pCMV6-XL5-E126D (E126D APE1) plasmid constructs over a six day period incubated at indicated concentrations of sodium arsenite. A single biological replicates is shown for each concentration of sodium arsenite (n = 1). All biological replicates displayed this growth pattern.
A: 0 mM sodium arsenite; B: 0.5 mM sodium arsenite; C: 1.0 mM sodium arsenite.

Statistical analysis of three biological replicates of the 0 mM sodium arsenite experiments was undertaken using two way ANOVA. A post-test analysis comparing the growth rate of wild-type APE1 HepG2 cells to the L104R and E126D APE1 HepG2 cells at each time point was performed. There were no statistical differences in the growth rates of wild-type APE1 HepG2 cells as compared to the growth rates of the L104R and E126D APE1 HepG2 cells at 0 hours, 24 hours and 48 hours (Figure 11). However, at 120 hours ($P < 0.05$) and 144 hours ($P < 0.05$), L104R APE1 HepG2 cells grew significantly faster compared to wild-type APE1 HepG2 cells (Figure 11). E126D APE1 HepG2 cell growth rates were observed to be significantly faster as compared to wild-type APE1 HepG2 cells at 72 hours ($P < 0.001$), 96 hours ($P < 0.01$) and 120 hours ($P < 0.01$) (Figure 11). These values match with the qualitative findings shown in Panel A, Figure 10. Thus, the qualitative trend of enhanced growth for HepG2 cells upon over-expression of L104R or E126D APE1 as compared to cells over-expressing wild-type APE1 was

statistically quantifiable, providing evidence that the L104R and E126D APE1 variants can facilitate the growth of HepG2 cells.

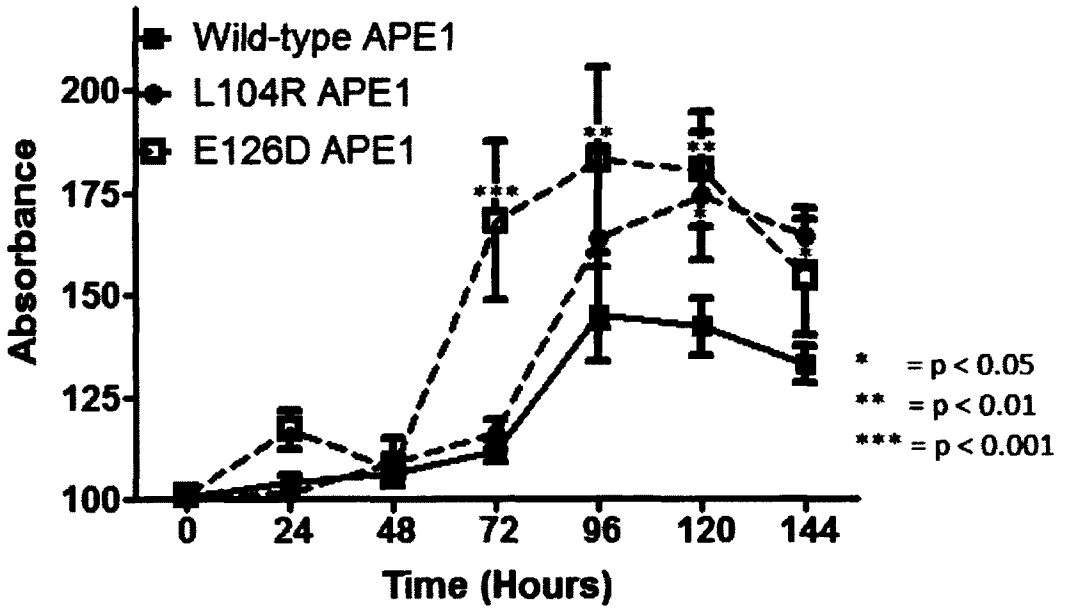

Figure 11. Growth of HepG2 cells transfected with pCMV6-XL5-Wild-typeAPE1 (Wildtype APE1), pCMV6-XL5-L104R (L104R APE1) or pCMV6-XL5-E126D (E126D APE1) plasmid constructs over a six day period. Absorbance values were normalized using the pCMV6-XL5 (Empty Vector) transfected HepG2 absorbance values at each time point. No sodium arsenite was added to the wells prior to incubation. Analysis performed was two way ANOVA with post-test analysis ($n = 3$).

Dot blot analyses were performed to determine whether the exogenous wild-type APE1, L104R APE1 and E126D APE1 proteins were indeed over-expressed in HepG2 cells. The initial dot blot performed contained proteins from the empty vector, wild-type APE1 HepG2 cells, and L104R APE1 HepG2 cells. Analysis of blot confirmed that, for both wild-type HepG2 cells and L104R APE1 HepG2 cells, there was a qualitative, approximately 2-fold increase in intensity of detectable APE1 protein as compared to empty vector HepG2 cells at all dilutions of the protein (Figure 12, 25 μg to 1.25 μg). In contrast, there was no difference in β-Actin levels in the

samples (Figure 12), demonstrating equal loading of protein samples. The second dot blot performed contained proteins isolated from the empty vector, wild-type APE1 HepG2 cells, and E126D APE1 HepG2 cells. Analysis of this blot confirmed over-expression of APE1 for wild-type APE1 HepG2 cells in addition to demonstrating an increase in APE1 protein levels for the E126D APE1 HepG2 cells at all loaded concentrations (Figure 13, 50 μg to 1.25 μg). In addition, the timing for the isolation of these proteins (72 hours) matched the first noticeable increase in cellular growth upon the over-expression of the population variants (Figure 10A).

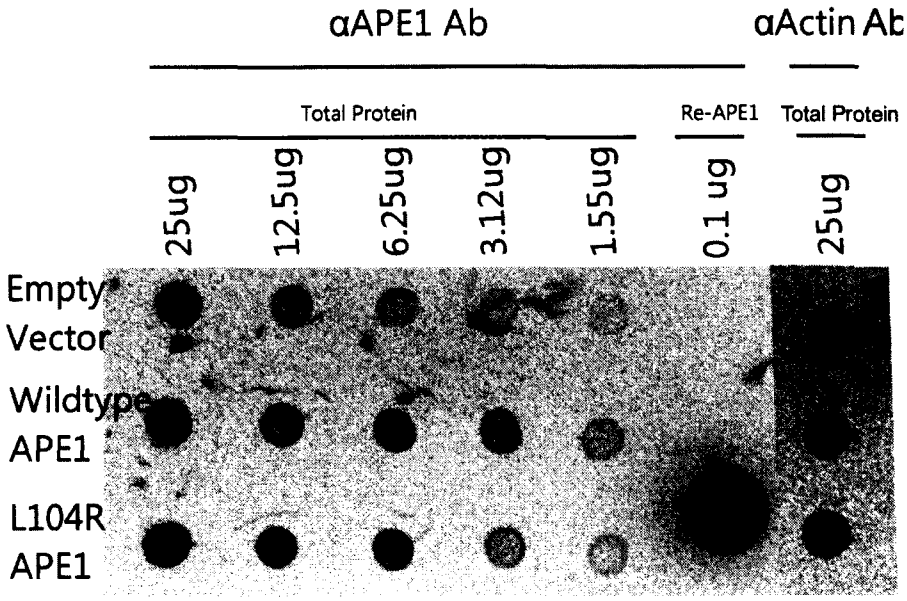

Figure 12. Dot blot of total cellular protein isolated from pCMV6-XL5-Vector (Empty Vector), pCMV6-XL5-Wild-typeAPE1 (Wild-type APE1) and pCMV6-XL5-L104R (L104R APE1) transfected HepG2 cells. Blots were subjected to incubation with either anti-human APE1 or anti-human β-actin monoclonal antibodies. Proteins were collected 72 hours after transfection and 0.1 μg of recombinant human APE1 was used as a positive control (n = 1).

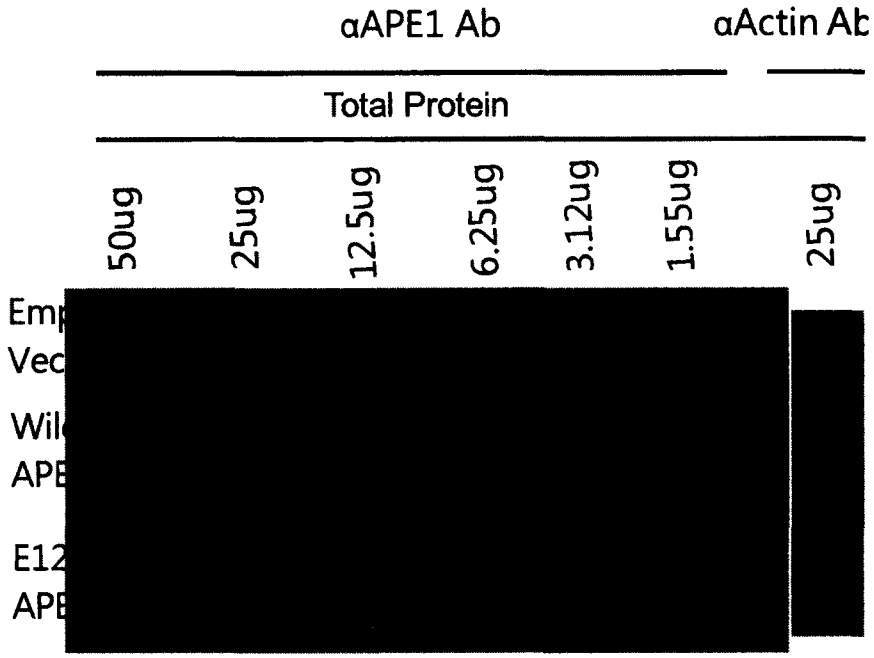

Figure 13. Dot blot of total cell proteins isolated from pCMV6-XL5-Vector (Empty Vector), pCMV6-XL5-Wild-typeAPE1 (Wild-type APE1) and pCMV6-XL5-E126D (E126D) transfected HepG2 cells. Samples were subjected to incubation with either anti-human APE1 or anti-human β-actin monoclonal antibodies. Proteins were collected 72 hours after transfection (n = 1).

3.3.5 Microarray Analysis and Preliminary qRT-PCR

To investigate whether the expression of L104R and E126D APE1 in HepG2 cells affected downstream gene expression as compared to wild-type APE1, mRNA was collected from a single biological replicate of (n =1) empty vector HepG2 cells, wild-type APE1 HepG2 cells, and L104R APE1 HepG2 cells. The total RNA samples were sent to the Hospital for Sick Children in Toronto for microarray analysis. Table 8 lists several genes with altered expression levels (fold change of 1.6 or above) when comparing between the wild-type APE1 HepG2 cells and L104R APE1 HepG2 cells. Two of these genes, highlighted in this table, are implicated in cell growth. BCL2-related protein A1 (BCL2A1) was determined to have a fold decrease of 2.26 in L104R APE1 HepG2 cells as compared to wild-type APE1 HepG2 cells (Table 8). BCL2A1 is a regulator of apoptosis in mammalian cells (Zhang *et al.* 2000). RAS and EF-hand domain (RASEF) was shown to have a modest fold increase of 1.75 in L104R HepG2 cells as compared to wild-type APE1 HepG2 cells (Table 8). RASEF is a substituent member of the larger RAS GTPase superfamily and is implicated in cell proliferation (Shintani *et al.* 2007). Thus, the functions of these two genes, correlated with the initial microarray results, suggests APE1 may be regulating the growth of HepG2 cells through these genes, and that the population variants have an effect on the ability of APE1 to regulate their cellular mRNA levels. Microarray analyses were further investigated using qRT-PCR. In addition to these two genes of interest, a third gene (*c-myc*) was chosen for analyses as *c-myc* mRNA has been demonstrated to be regulated by wild-type APE1 in HeLa cells (Barnes *et al.* 2009).

Table 8. Microarray results with a fold change of greater than 1.6. Three transfection conditions were compared - pCMV6-XL5-Vector (Empty Vector), pCMV6-XL5-WildtypeAPE1 (Wildtype APE1) and pCMV6-XL5-L104R (L104R APE1). Highlighted genes were chosen for subsequent qRT-PCR analysis based on their growth related functions in eukaryotic cells. (n = 1).

Gene Identity	L104R APE1 vs Wild-type APE1	L104R APE1 vs EmptyVector	Wild-type APE1 vs Empty Vector
	Fold Increase		
Solute Carrier Family 6, member 12 (SLC6A12)	+1.96	-1.91	-3.76
Tensin 1 (TNS1)	+1.95	+1.34	-1.44
RAS and EF-hand domain (RASEF)	+1.75	+4.12	+2.35
WD repeat domain 61 (WDR61)	+1.74	+1.50	-1.15
Phosphoenolpyruvate Carboxykinase 1(PCK1)	+1.71	-1.01	-1.81
	Fold Decrease		
Collagen, type 1, alpha 1 (COL1A1)	-2.58	-1.67	+1.54
BCL2-related protein A1 (BCL2A1)	-2.26	-1.46	+1.58
Serologically defined colon cancer antigen 8 (SDCCAG8)	-1.78	n/a	n/a
Potassium Voltage-gated Channel, subfamily H (KCNH1)	-1.72	-1.16	+1.48
Troponin T type 1	-1.61	-1.27	+1.26

To confirm primer specificity and to determine an annealing temperature which would produce a single product, melt curves for these two genes were conducted at a range of temperatures. At all annealing temperatures tested (52°C to 62°C), both BCLA2 and RASEF primers produced a single peak which melted at approximately 86°C (Figure 14A) and 83°C (Figure 14B), respectively. This indicates a single product formation for these primers at these temperature ranges. Thus, any of the temperatures tested here can be used for downstream gene expression studies as the annealing temperature.

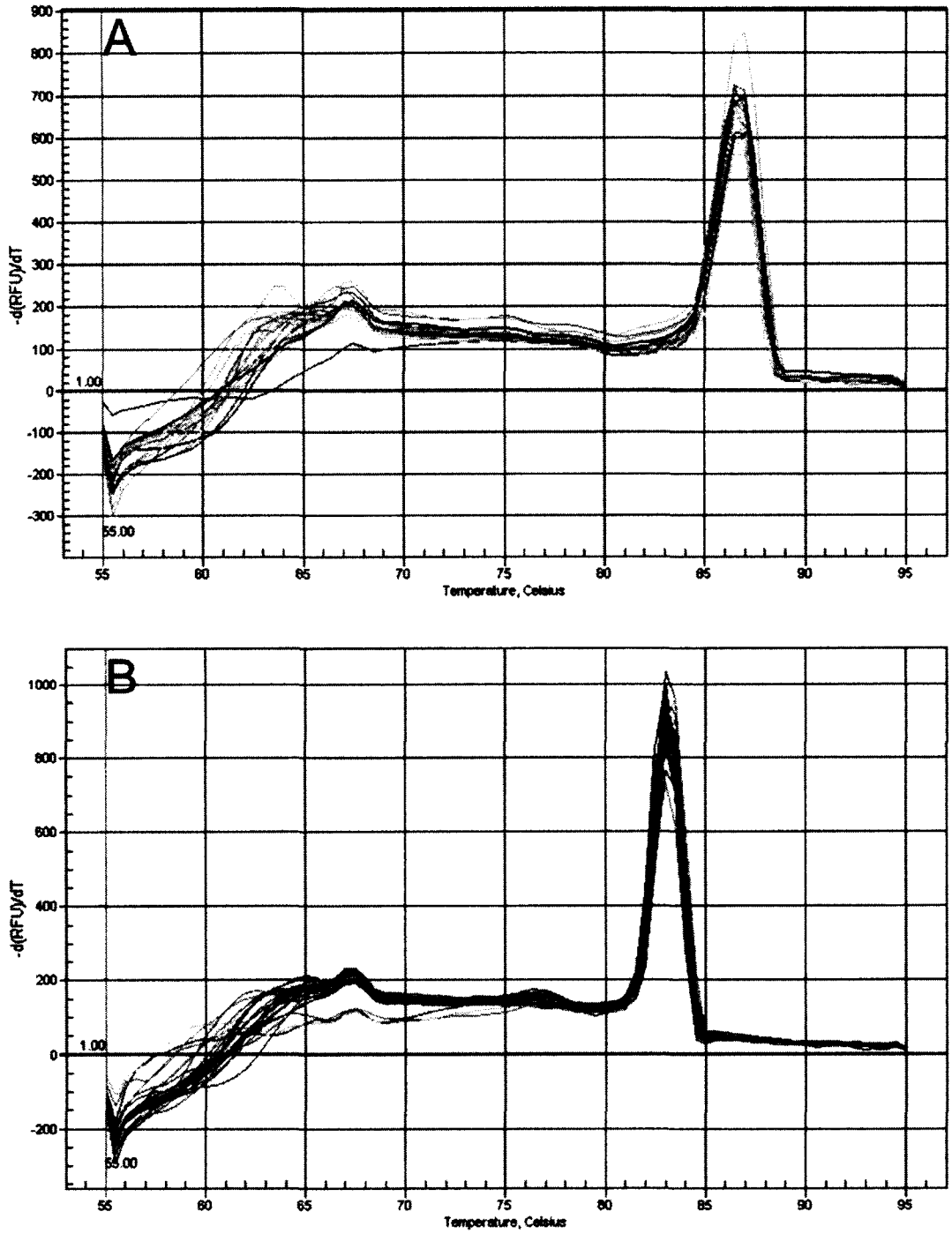

Figure 14. Melt curve analyses of the two primer sets used for downstream gene study applications. mRNA analyzed was pooled from mRNA isolated from HepG2 cells transfected with pCMV6-XL5-Vector, pCMV6-XL5-Wild-typeAPE1 and pCMV-XL5-L104R plasmid vectors.
A) BCL2A1 primers; B) RASEF primers

As previous experiments in Dr. Lee's lab had established that β-Actin and *c-myc* primers form a single product at an annealing temperature of 60°C (Kim *et al.* 2009), it was decided to proceed with an annealing temperature of 60°C for the subsequent qRT-PCR gene expression analyses. For the BCL2A1 gene, preliminary analysis using two biological replicates ($n = 2$) indicate that, when normalized to the β-Actin housekeeping gene, there was no statistical change in fold expression when comparing L104R APE1 HepG2 cells to that of wild-type APE1 HepG2 cells or empty vector HepG2 cells (Figure 15). In addition, preliminary analysis of the RASEF gene indicated no change in expression level when comparing L104R HepG2 cells to that of wild-type APE1 HepG2 cells or empty vector HepG2 cells for two biological replicates ($n = 2$) (Figure 15). These qRT-PCR results are contradictory with the microarray data, which demonstrated a decrease and increase in fold expression for the BCL2A1 and RASEF genes, respectively (Table 8). From a single biological replicate ($n = 1$), it was determined that L104R cells had no change in the expression levels of *c-myc* (Figure 15) as compared to wild-type APE1 cells.

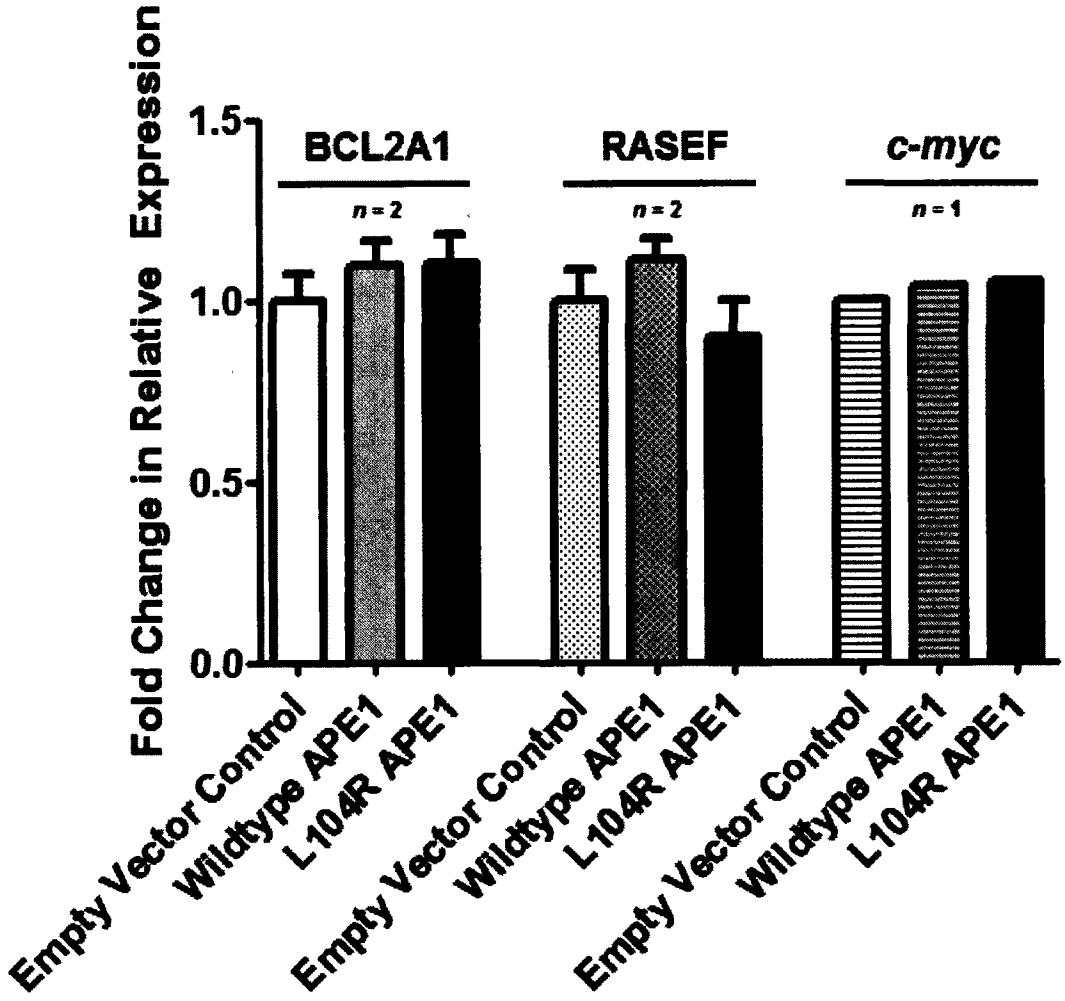

Figure 15. A summary of the quantitative real time polymerase (qRT-PCR) gene expression studies comparing the mRNA levels for select genes between the pCMV6-XL5-Vector (Empty Vector Control), pCMV6-XL5-Wild-typeAPE1 (Wild-type APE1) and pCMV6-XL5-L104R (L104R APE1) transfected cells. Gene expression studies were normalized using the β-Actin gene.

3.4 Discussion

3.4.1 Effects on Growth Rate of HeLa Cells upon Knockdown of APE1 or Over-expression of APE1 Population Variants

The first step in the eukaryotic cell investigations was to determine if APE1 is important in the growth of HeLa cells. A change in growth phenotype was expected, as a previous study had demonstrated that the knock down of APE1 in HeLa cells resulted in an increase in the steady state levels of mRNA for a powerful growth related gene, *c-myc* (Barnes *et al.* 2009). However, the knockdown APE1 HeLa cells demonstrated identical, qualitative, growth rate as compared to the control HeLa cells (Figure 6, all panels). Such results suggest that endogenous APE1 does not appear to play an important role in the modulation of growth rate in HeLa cells. However, the results from Chapter 2 (observed increase in endoribonuclease activity of the L104R and E126D APE1 variants in Origami™ (DE3) cells) together with the observations of altered RNA cleavage pattern and the RNasin resistant characteristics of these variants *in vitro* (Kim *et al.* 2012), suggest a gain of function for the L104R and E126D APE1 variants. This may not be observed in a system where APE1 levels are being lowered and may only be observed if the variants were over-expressed. Thus, it was deemed logical to conduct experiments to over-express the L104R and E126D APE1 population variants and assess their effect upon the growth rates of mammalians cells such as HeLa cells.

The growth rates of L104R APE1 HeLa cells and E126D APE1 HeLa cells were quantitatively comparable to both wild-type APE1 HeLa cells and empty vector HeLa cells (Figure 7, all panels). These results did not match the hypothesis which predicted that there would be differences in growth rates between the L104R and E126D APE1 cells as compared to

the wild-type APE1 cells. Several reasons could possibly explain for this lack of effect of growth of HeLa cells by these APE1 variants. One possible explanation involves the plasmid used. The particular plasmid constructs used for these experiments were the pCMV5.1-APE1-FLAG plasmid constructs and thus all contained a FLAG tag. The FLAG tag may have interfered with any effects of the protein folding or substrate binding of the APE1 variant proteins. Thus, it was decided to use a new vector (PCMV6-XL5) which removed the 3'UTR of APE1 and the FLAG tag for downstream experiments. In addition, concurrent research in Dr. Lee's lab had initially demonstrated that cellular localization of APE1 in HepG2 cells may be dependent on cellular stress. Specifically, APE1 was localizing to P-bodies in the cytoplasm upon stress with sodium arsenite. Therefore we hypothesized that any altered mRNA regulation driven by these APE1 variants may only be observed if APE1 were localized to these P-bodies, as P-bodies have been shown to be the sites for mRNA degradation (Garneau *et al.* 2007). Thus, subsequent growth rate experiments incorporated sodium arsenite stress. Finally, it may be possible that APE1 simply does not play any significant role in growth modulation in HeLa cells. Thus, a human hepatoma cancer cell line, HepG2, was chosen for subsequent experiments as APE1 may possibly play a different role in growth modulation for these cells as compared to HeLa cells.

In summary, neither APE1 nor the L104R or E126D variants of APE1 play any role in the modulation of growth rates in HeLa cells.

3.4.2 Effects on Growth Rate of HepG2 Cells upon Knockdown of APE1 or Over-expression of APE1 Population Variants

We next examined the effects on growth rate of HepG2 cells upon knock down of APE1. Knockdown APE1 HepG2 cells showed a qualitative decrease in growth rate as compared to the control HepG2 cells for all concentrations of sodium arsenite tested (Figure 8, all panels). These

results demonstrated that endogenous APE1 plays a role in facilitating the growth of HepG2 cells. The cause for this decrease in cellular growth rate, however, cannot be determined from this experiment. An absence of any of the known functions of APE1 (Section 1.3) may contribute to this observed decrease in growth rate. However, the results here agree with previous observations where APE1 has been knocked down: APE1 was determined, using a proteomics approach, to be important for many functions including cell growth, apoptosis, intracellular redox state and mitochondrial function (Vascotto *et al.* 2009).

The over-expression of L104R and E126D APE1 proteins resulted in a qualitatively enhanced growth as compared to that of wild-type APE1 HepG2 cells and empty vector HepG2 cells in the absence or presence of 0.5 mM sodium arsenite (Figure 10, Panels A and B). There was less of a qualitative difference between L104R and E126D APE1 HepG2 cells compared to wild-type APE1 HepG2 cells and empty vector HepG2 cells at 1.0 mM sodium arsenite. This may be explained by the fact that sodium arsenite was toxic to the cells. There is a visible, qualitative decrease in cellular growth for the 1.0 mM sodium arsenite incubation which is in contrast to the overall growth of cells in 0 mM sodium arsenite (Figure 10C versus Figure 10A). This may interfere with any possible enhancement of growth rate by the population variants for this particular incubation condition as the cells were not growing under such toxic conditions. A more detailed analysis of the growth rate of HepG2 cells upon expression of the APE1 population variants was carried out using two-way ANOVA analysis of data pooled from three biological replicates. The results were mixed, with statistical significance only being present for the L104R APE1 HepG2 cells at 120 and 144 hours while the E126D APE1 HepG2 cells showed statistical significance at 72, 96 and 120 hours (Figure 11). This suggests the increased growth effects of expressing these variants were modest. However, there was some evidence indicating

the increased growth rate observed in these growth assays may be biologically relevant. First, the L104R APE1 HepG2 and E126D APE1 HepG2 cells consistently demonstrated an increase in growth rate as compared to wild-type APE1 HepG2 cells over multiple biological replicates. Furthermore, there are examples in the literature which stated that changes in growth rate are translatable to disease. For example, at 96, 120, and 144 hours there was approximately 130-150% increased growth for the L104R and E126D APE1 HepG2 cells as compared to that of wild-type APE1 HepG2 cells (Figure 11). This is comparable to the 150% increase in cell growth observed in a study linking over-expression of Vascular Endothelial Growth Factor (VEGF) to stimulation of angiogenesis and progression of ovarian cancer (Duyndam *et al.* 2002). Hence, the increase in growth rate observed in these experiments may translate directly into biological relevancy in terms of disease pathogenesis.

For the HepG2 growth assays, sodium arsenite was employed to stress the cells as concurrent research in Dr. Lee's lab had shown that APE1 may localize to P-bodies in the cytoplasm under stress. Subsequent work has demonstrated that this is not the case and that localization of APE1 in HepG2 cells is not affected by sodium arsenite-induced stress. This appears to agree with the results obtained here as mild sodium arsenite stress (0.5 mM) did not alter the enhanced growth of L104R and E126D APE1 HepG2 cells as compared to no sodium arsenite treatment (Figures 10 and 11). These results suggests that sodium arsenite has no direct influence on the ribonuclease activity of APE1 or its variants and that APE1 and its population variants play roles in the growth of HepG2 cells regardless of cellular stress levels.

The results of the over-expression of L104R and E126D APE1 proteins on the growth of HepG2 cells (enhanced cell growth for the L104R and E126D APE1 HepG2 cells in 0 mM sodium arsenite) as well as the demonstration of exogenous mutant APE1 protein expression for

these population variants (Figure 12; Figure 13), showed a correlation between over-expression of the L104R and E126D APE1 proteins and enhanced cellular growth of HepG2 cells. Both the L104R and E126D APE1 variants have exhibited altered RNA cleaving activity as compared to the wild-type APE1 *in vitro* (Kim *et al.* 2012); therefore we elected to investigate if these variants have any effect on gene expression through altered ribonuclease activity.

3.4.3 Comparing the Effects of APE1 and its Population Variants on HeLa and HepG2 cells

The knockdown of APE1 and over-expression of APE1 population variants in HeLa cells showed no change in growth rate (Figures 6; Figure 7). In contrast, the knockdown of APE1 and over-expression of APE1 population variants in HepG2 cells showed a decrease and increase in growth rates respectively (Figures 8; Figure 10). This poses an interesting question: why are growth rate changes observable only for one cell line? It is possible that the observed changes for HepG2 cells as compared to that of HeLa cells may be explained by the following facts: 1) HepG2 cells are hepatoma cancer cells with advanced differentiation while HeLa cells are cervical cancer cells with relatively less differentiation; 2) APE1 is known to have distinct localization patterns in differing tissue types (Tell *et al.* 2005). As hepatoma cells are not cervical cells, it is possible that APE1 and its population variants play a different role in the modulation of growth in these two cell lines. In addition, the mechanisms which lead to a cancer phenotype in these cells are not identical and thus may have distinct altered signaling pathways. Thus, it is possible that APE1 and its population variants are only relevant to a molecular growth signal present in HepG2 cells but not in HeLa cells.

To summarize, it was observed that for HeLa cells, both the knockdown of APE1 and over-expression of the population variants resulted in no observable changes in growth. However, the knockdown of APE1 and over-expression of the population variants in HepG2 resulted in an observable decrease and increase in growth rate, respectively. The next set of gene expression experiments were performed to potentially determine the molecular mechanism whereby APE1 and its population variants caused the observed change in growth of HepG2 cells

3.4.4 Microarray Analysis and Preliminary qRT-PCR

This section of the thesis was to determine if the altered RNA cleaving function of the L104R and E126D APE1 population variants was responsible for the observed change in growth rate of HepG2 cells. To this end, we elected to over-express the APE1 population variant L104R in HepG2 cells, harvest mRNA from the L104R APE1 HepG2 cells and subsequently analyze harvested mRNA for gene expression changes using microarray analysis. Microarray analysis was chosen as it would provide information into the possible molecular targets of this APE1 population variant. If these targets were determined to be growth related genes, then a strong correlation between the APE1 population variants targeting growth related genes and the observed increase in growth upon over-expressing the variants in HepG2 cells would be demonstrated. The preliminary microarray experiment showed that two growth related genes had altered expression levels in L104R APE1 HepG2 cells as compared to that of wild-type APE1 HepG2 cells. The first was BCL2-related protein A1 (BCL2A1) which is known to act as a regulator of apoptosis in mammalian cells (Zhang *et al.* 2000). The second was RAS and EF-hand domain (RASEF), which is a substituent member of the larger RAS GTPase superfamily and is implicated in cell proliferation (Shintani *et al.* 2007) (Table 8). This was evidence that the initial hypothesis presented for this section was possibly true. However, we recognized some

flaws in the present preliminary microarray analysis: 1) the low number of biological repeats (*n* = 1) and 2) microarray data is known to contain background noise. Preliminary qRT-PCR was therefore performed in an effort to confirm the results from the microarray analysis.

In addition to the BCL2A1 and RASEF genes, analysis was carried out for the *c-myc* gene, as *c-myc* mRNA levels are regulated by APE1 in HeLa cells (Barnes *et al.* 2009). Unfortunately, the results of the qRT-PCR experiments did not match the results obtained from the microarray data. From two biological replicates, it was determined that the L104R-expressing HepG2 cells did not have any change in the expression levels of BCL2A1 or RASEF mRNA (Figure 15). There was also no observable effect on *c-myc* mRNA levels from a single biological replicate (Figure 15). It is possible that the qRT-PCR results are more valid than the microarray data as: 1) for the BCL2A1 and RASEF genes, there were two biological replicates performed as compared to just one for the microarray analysis study; and 2) the melt curves performed for the primers used in this experiment showed specificity of the primers. However, the qRT-PCR results may not be reliable either. The protocols for the qRT-PCR in this thesis do not conform to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. RNA quality control was performed by comparing the A_{260}/A_{280} ratio for all RNA samples isolated against the established ratio of 1.8-2.0 for pure RNA in lieu of assessing RNA integrity by determining the 28S:18S rRNA ratio. Electrophoretic assays to assess for the 28S:18S rRNA ratio of 2.7:1, the standard for RNA quality assessment, was not performed. In addition, only a single reference gene was used for normalization, which is in contrast to the MIQE guidelines, which require multiple reference genes. As such, the conclusion drawn in this thesis, regarding the gene expression studies, are preliminary results which will need to be verified in future studies if this project is to be pursued.

We tentatively conclude that the population variant L104R does not specifically regulate the expression of the BCL2A1, RASEF or *c-myc* genes leading to an altered growth phenotype in HepG2 cells. However, we do not rule out the possibility of altered mRNA cleaving function of this variant as a cause for the change in growth in HepG2 cells. It is entirely possible that there are yet unidentified molecular targets of the L104R APE1 variant which is responsible for the change in growth *via* mRNA cleavage. However, we also do not rule out the possibility that the change in growth phenotype is due to a deregulation of the other known functions of APE1.

Chapter 4

General Discussion

4.1 General Overview

APE1 is a multifunctional protein implicated in many important cellular roles including abasic DNA endonuclease cleavage (Evans *et al.* 2000), acting as a redox factor for many growth-related transcription factors (Evans *et al.* 2000), 3'-5' exonuclease activity for DNA (Chou *et al.* 2000), and endoribonuclease activity against both mRNA and abasic RNA *in vitro* (Barnes *et al.* 2009; Berquist *et al.* 2008). APE1 is also known to exist as multiple population variants (Section 1.4.1). Previously, some of these population variants have been linked to diseases (Section 1.4.2). For instance, the L104R and E126D APE1 variants have been previously linked with ALS, and the D148E APE1 variant with various cancers (Section 1.4.2). To date, it is still unknown if alterations to of any of the known biochemical functions exhibited by the APE1 population variants has any impact at the cellular or organismal level, or whether there is a direct influence on susceptibility to disease. Investigation into the ribonuclease activity of the L104R and E126D APE1 variants has shown that they demonstrate altered cleavage patterns on *c-myc* CRD mRNA *in vitro* as compared to that of wild-type APE1 (Table 1). In addition, these variants were shown to be resistant to the effects of a powerful RNase inhibitor, RNasin (Kim *et al.* 2012). In contrast, the D148E APE1 variant demonstrated complete abrogation of RNA cleaving activity against *c-myc* CRD mRNA (Kim *et al.* 2012).

4.2 Population Variants of APE1 and Disease

One of the goals of this investigation was to examine if over-expression of the APE1 population variants L104R, E126D and D148E would result in a change in phenotype in prokaryotic and eukaryotic cells and thus suggest a biochemical correlation between this altered phenotype and the diseases they are associated with.

This thesis demonstrated that the L104R and E126D APE1 variants were cytotoxic to Origami™ (DE3) cells while stimulating the growth of HepG2 cells when over-expressed. The results here may or may not represent a link between the L104R and E126D variants and ALS. ALS is characterized by the degeneration of neuronal cells (Deng *et al.* 2011). If the results of the Origami™ (DE3) cell experiments are taken into account, then it is possible that these variants may confer an increase in cytotoxicity to neuronal cells expressing these variants and may therefore be involved in the degeneration of the cells. This could be of particular interest as a new avenue of investigation into the pathogenesis of ALS. Most contemporary studies implicate the causes of ALS to mutations of the SOD1 gene (Al-Chalabi *et al.* 2000) and environmental factors such as head trauma (Piazza *et al.* 2004). Our results of the mammalian cell data is contradictory to the prokaryotic data as the L104R and E126D APE1 variants demonstrate either no effect on the growth of HeLa cells or enhanced growth of HepG2 cells (Section 3.3.2, Section 3.2.4).

Since ALS is attributed to the degeneration of cells, the observed enhanced growth implies that the above variants may not be responsible for the cell death observed in ALS. However, the mammalian data is likely to be a more accurate representation of the situation in ALS as ALS is a mammalian disease. Also, it must be considered that the APE1 protein plays

distinct roles in differing cell types due to altered localization patterns (Tell *et al.* 2005). Since hepatoma cells are not neuronal cells, it is possible that the expression of these variants in neuronal cells may result in cell death as opposed to the enhanced hepatoma cell growth in this thesis. In addition, this thesis did not elucidate the biochemical functions of APE1 which are responsible for the enhanced cell growth in HepG2 cells. Thus, any of the functions of APE1 could be implicated in ALS. Therefore, this thesis cannot draw a biochemical link between the expression of the L104R and E126D APE1 variants and ALS. To investigate the possible link between these variants and the pathogenesis of ALS, future experiments should be performed using mammalian neuronal cells and observing phenotypic changes such as cell survivability. If changes in growth phenotypes are observed, then gene expression studies should be pursued with a focus on selecting appropriate reference genes and controlling RNA quality.

The D148E APE1 variant has been found to be associated with a large variety of cancers (Section 1.4.3). The hypothetical cause for the association of this variant with cancer was proposed in this thesis to be disruptions to the ribonuclease activity of APE1. Previously, *in vitro* work had shown a complete loss of RNA cleaving activity for this variant against *c-myc* CRD RNA (Kim *et al.* 2012). However, the results of this thesis showed that the D148E APE1 variant had no effect on cell growth in a prokaryotic system (Section 2.3). This suggests that the hypothesis linking deregulation of the endoribonuclease activity of D148E APE1 to cellular function does not hold true. Further studies of this variant on mammalian cell systems have not been performed. Given that the sensitivity of the prokaryotic system may have been inadequate to detect a subtle loss of function of ribonuclease activity of proteins (Section 2.4), it may be worthwhile for future studies to assess D148E APE1 in mammalian systems including in neuronal cells. This, in itself, may prove to be difficult because the D148E variant is likely

responsible for disease in a loss of function aspect. In such a scenario, mammalian cell lines used for studies must have an absence of endogenous APE1. This is due to the fact that endogenous APE1 would mask the effects of a loss of function in the D148E APE1 variant. Hence, the most appropriate model would be cells that have dominant negative D148E mutations.

4.3 Concluding Remarks

Several studies have identified human population variants of APE1. Some of these known population variants (L104R, E126D and D148E, in particular) have been correlated with diseases such as ALS and cancer. To date, there have been no studies linking the ribonuclease activity of the population variants of APE1 to disease. The main goals of this MSc thesis were to test the hypothesis that these population variants, when expressed in cells, would have an effect on phenotype and that this effect could be linked to their ribonuclease activity. The results of the Origami™ (DE3) cell experiments indicated that L104R and E126D APE1 variants have an inhibitory effect on the growth of cells. Also, the experiments suggest that this effect is due to ribonuclease activity as these cells have been previously used to demonstrate cytotoxicity, through ribonuclease activity, in similar experiments conducted for the ribonucleases RNase A and Angiogenin (Smith *et al.* 2006; Smith *et al.* 2008). Subsequent expression of the L104R and E126D APE1 variants in mammalian cells demonstrated a stimulatory effect on the growth of HepG2 cells; however, attempts to link this effect to their ribonuclease activity were unsuccessful. It is possible that any of the several known functions of APE1 and its population variants were responsible for this effect. In addition, it was found that APE1 and its population variants displayed growth effects on HepG2 cells but not on HeLa cells. It is possible that APE1 plays a different role in various cell types by interacting with multiple unique targets in a given cell type. Thus, future studies should include: 1) Investigating the biochemical function(s) of

APE1 responsible for stimulating growth in HepG2 cells; 2) Investigating the molecular targets of APE1 (for each biochemical function of APE1) in several different cell lines, particularly in neuronal cells. The initial tentative findings here give credence to the hypothesis that there is a link between endoribonuclease activity and cellular phenotype for the APE1 population variants, but future studies will need to be performed to clarify our further understanding of the issue.

References

Abate, C., Patel, L., Rauscher, F.J. and Curran, T. (1990) Redox regulation of fos and jun DNA-binding activity in vitro. *Science*, **249**, 1157-1161.

Agaçhan, B., Küçükhüseyin, O., Aksoy, P., Turna, A., Yaylim, I., Görmüs, U., Ergen, A., Zeybek, U., Dalan, B. and Isbir, T. (2009) Apurinic/apyrimidinic endonuclease (APE1) gene polymorphisms and lung cancer risk in relation to tobacco smoking. *Anticancer Res*, **29**, 2417-2420.

Al-Attar, A., Gossage, L., Fareed, K.R., Shehata, M., Mohammed, M., Zaitoun, A.M., Soomro, I., Lobo, D.N., Abbotts, R., Chan, S. and Madhusudan, S. (2010) Human apurinic/apyrimidinic endonuclease (APE1) is a prognostic factor in ovarian, gastro-oesophageal and pancreatico-biliary cancers. *Br J Cancer*, **102**, 704-709.

Al-Chalabi, A. and Leigh, P.N. (2000) Recent advances in amyotrophic lateral sclerosis. *Curr Opin Neurol*, **13**, 397-405.

Ando, K., Hirao, S., Kabe, Y., Ogura, Y., Sato, I., Yamaguchi, Y., Wada, T., Handa, H. (2008) A new APE1/Ref-1-dependent pathway leading to the reduction of NF-kappaB and AP-1, and activation of their DNA-binding activity. *Nucleic Acids Res*, **36**, 4327-4336.

Audic, Y. and Hartley, R.S. (2004) Post-transcriptional regulation in cancer. *Biol Cell,* **96**, 479-498.

Baldwin, M.R. and O'Brien, P.J. (2009) Human AP endonuclease 1 stimulates multiple-turnover base excision by alkyladenine DNA glycosylase. *Biochemistry*, **48**, 6022-6033.

Barnes, T., Kim, W.C., Mantha, A.K., Kim, S.E., Izumi, T., Mitra, S. and Lee, C.H. (2009) Identification of Apurinic/apyrimidinic endonuclease 1 (APE1) as the endoribonuclease that cleaves c-myc mRNA. *Nucleic Acids Res,* **37,** 3946–3958.

Bassett, T.A. 2007. Assessing the role of Syntaxin 18 in controlling c-myc mRNA expression and growth in human breast cancer cells [M.Sc. Thesis]. Prince George, University of Northern British Columbia.

Beernink, P.T., Segelke, B.W., Hadi, M.Z., Erzberger, J.P., Wilson, D.M. III. (2001) Two divalent metal ions in the active site of a new crystal form of human apurinic/apyrimidinic endonuclease, Ape1: implications for the catalytic mechanism. *J Mol Biol*, **307**, 1023-1034.

Behm-Ansmant, I., Kashima, I., Rehwinkel, J., Sauliere, J., Wittkopp, N. ans Izaurralde, E. (2007) mRNA quality control: an ancient machinery recognizes and degrades mRNAs with nonsense codons. *FEBS Lett,* **581**, 2845–2853.

Belostotsky, D. (2004) mRNA turnover meets RNA interference. *Mol Cell*, **16**, 498-500.

Bernstein, E., Kim, S.Y., Carmell, M.A., Murchison, E.P., Alcorn, H., Li, M.Z., Mills, A.A., Elledge, S.J., Anderson, K.V. and Hannon, G.J. (2003) Dicer is essential for mouse development. *Nat Genet,* **35**, 215–217.

Berquist, B.R., McNeill, D.R., and Wilson, D.M. III. Characterization of abasic endonuclease activity of human APE1 on alternative substrates, as well as effects of ATP and sequence context on AP site incision. *J Mol Biol*, **379**, 17-27.

Bisbal, C., Silhol, M., Laubenthal, H., Kaluza, T., Carnac, G., Milligan, L., Le Roy, F. and Salehzada, T. (2000) The 2′-5′ oligoadenylate/RNase L/RNase L inhibitor pathway regulates both MyoD mRNA stability and muscle cell differentiation. *Mol Cell Biol*, **20**, 4959–4969.

Bracken, C.P., Szubert, J.M., Mercer, T.R, Dinger, M.E, Thomson, D.W., Mattick, J.S., Michael, M.Z. and Goodall, G.J. (2011) Global analysis of the mammalian degradome reveals widespread miRNA-dependent and miRNA-independent endonucleolytic cleavage. *Nucleic Acids Res*, **39**, 5658-5668.

Breton, C.V., Zhou, W., Kile, M.L., Houseman, E.A., Quamruzzaman, Q., Rahman, M., Mahiuddin, G., Christiani, D.C. (2007) Susceptibility to arsenic-induced skin lesions from polymorphisms in base excision repair genes. *Carcinogenesis*, **28**, 1520-1525.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. and Wittwer, C.T. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Che*, **55**, 611-622.

Calfon, M., Zeng, H., Urano, F., Till, J.H., Hubbard, S.R., Harding, H.P., Clark, S.G. and Ron, D. (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature*, **415**, 92–96.

Canbay, E., Agachan, B., Gulluoglu, M., Isbir, T., Balik, E., Yamaner, S., Bulut, T., Cacina, C., Eraltan, I.Y., Yilmaz, A. and Bugra, D. (2010) Possible associations of APE1 polymorphism with susceptibility and HOGG1 polymorphism with prognosis in gastric cancer. *Anticancer Res*, **30**, 1359-1364.

Canbay, E., Cakmakoglu, B., Zeybek, U., Sozen, S., Cacina, C., Gulluoglu, M., Balik, E., Bulut, T., Yamaner, S. and Bugra, D. (2011) Association of APE1 and hOGG1 polymorphisms with colorectal cancer risk in a Turkish population. *Curr Med Res Opin*, **7**, 1295-1302.

Canete-Soler, R., Reddy, K.S., Tolan, D.R. and Zhai, J. (2005) Aldolases a and C are ribonucleolytic components of a neuronal complex that regulates the stability of the light-neurofilament mRNA. *J Neurosci,* **25**, 4353–4364.

Chang, Y.F., Imam, J.S. and Wilkinson, M.F. (2007) The nonsense-mediated decay RNA surveillance pathway. *Annu Rev Biochem*, **76**, 51-74.

Chang, J.S., Wrensch, M.R., Hansen, H.M., Sison, J.D., Aldrich, M.C., Quesenberry, C.P. Jr., Seldin, M.F., Kelsey, K,T. and Wiencke, J.K. (2009) Base excision repair genes and risk of lung cancer among San Francisco Bay Area Latinos and African-Americans. *Carcinogenesis*, **30**, 78-87.

Chattopadhyay, R., Das, S., Maiti, AK., Boldoghm I., Xie, J., Hazra, T.K., Kohno, K., Mitra, S. and Bhakat, K.K. (2008) Regulatory role of human AP-endonuclease (APE1/Ref-1) in YB-1-mediated activation of the multidrug resistance gene MDR1. *Mol Cell Biol,* **28**, 7066-80.

Chen, L., Ambrosone, C.B., Lee, J., Sellers, T.A., Pow-Sang, J. and Park, J.Y. Association between polymorphisms in the DNA repair genes XRCC1 and APE1, and the risk of prostate cancer in white and black Americans. *J Urol*, **175**, 108-112.

Chiang, F.Y., Wu, C.W., Hsiao, P.J., Kuo, W.R., Lee, K.W., Lin, J.C., Liao, Y.C., Juo, S.H. (2008) Association between polymorphisms in DNA base excision repair genes XRCC1, APE1, and ADPRT and differentiated thyroid carcinoma. *Clin Cancer Res*, **14**, 5919-5924.

Chou, K.M., Kukhanova, M. and Cheng, Y.C. (2000) A novel action of human apurinic/apyrimidinic endonuclease: excision of L-configuration deoxyribonucleoside analogs from the 3' termini of DNA. *J Biol Chem*, **275**, 31009-31015

Cunningham, K.S., Hanson, M.N. and Schoenberg, D.R. (2001) Polysomal ribonuclease 1 exists in a latent form on polysomes prior to estrogen activation of mRNA decay. *Nucleic Acids Res,* **29**, 1156–1162.

Curtis, C.D., Thorngren, D.L., Ziegler, Y.S., Sarkeshik, A., Yates, J.R. and Nardulli, A.M. (2009) Apurnic/Apurimidinic Endonuclease 1 Alters Estrogen Receptor Activity and Estrogen Responsive Gene Expression. *Mol Endocrinol*, **23**, 1346-59.

Di Maso, V., Avellin,i C., Crocè, L.S., Rosso, N., Quadfrifoglio, F., Cesaratto, L., Codarin, E., Bedogni, G., Beltrami, C.A., Tell, G. and Tiribelli, C. (2007) Subcellular localization of

APE1/Ref-1 in human hepatocellular carcinoma: possible prognostic significance. *Mol Med*, **13**, 89-96.

Duyndam, M.C., Hilhorst, M.C., Schlüper, H.M., Verheul, H.M., van Diest, P.J., Kraal, G., Pinedo, H.M. and Boven, E. (2007) Vascular endothelial growth factor-165 overexpression stimulates angiogenesis and induces cyst formation and macrophage infiltration in human ovarian cancer xenografts. *Am J Pathol*, **160**, 537-548.

Eberle, A.B., Lykke-Andersen S., Muhlemann, O. and Jensen, T.H. (2009) SMG6 promotes endonucleolytic cleavage of nonsense mRNA in human cells. *Nat Struct Mol Biol*, **16**, 49-55.

Evans, A.R., Limp-Foster, M., Kelley, M.R. (2000) Going APE over ref-1. *Mut Res 2000*, **461**, 83-108.

Fan, J., Yang, X, Wang, W., Wood III W.H., Becker, K.G. and Gorospe M. (2002) Glocal analysis of stress-regulated mRNA turnover by using cDNA arrays. *Proc Natl Acad Sci USA*, **99**, 10611-10616.

Fantini, D., Vascotto, C., Deganuto, M., Bivi, N., Gustincich, S., Marcon, G., Quadrifologlio, F., Damante, G, Bhakat, K.K., Mitra, S. and Tell, G. (2008) APE1/REF-1 regulates PTEN expression mediated by Egr-1. *Free Radic Res*, **42**, 20-29.

Fett, J.W., Strydom, D.J., Lobb, R.R., Alderman, E.M., Bethune, J.L., Riordan, J.F. and Vallee, B.L. (1985) Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. *Biochemistry,* **24**, 5480–5486.

Fishel, M.L. and Kelley, M.R. (2007) The DNA base excision repair protein APE1/Ref-1 as a therapeutic and chemopreventive target. *Molecular Aspects of Medicine*, **28**, 375-395.

Gallouzi, I.E., Parker, F., Chebli, K., Maurier, F., Labourier, E., Barlat, I., Capony, J.P., Tocque, B. and Tazi, J. (1998) A novel phosphorylation-dependent RNase activity of GAP-SH3 binding protein: a potential link between signal transduction and RNA stability. *Mol Cell Biol*, **18**, 3956–3965.

Gangwar, R., Ahirwar, D., Mandhani, A. and Mittal, R.D. (2009) Influence of XPD and APE1 DNA repair gene polymorphism on bladder cancer susceptibility in north India. Urology, **73**, 675-680.

Garneau, N.L., Wilusz, J. and Wilusz, C.J. (2007) The highways and byways of mRNA decay. *Nat Rev Mol Cell*, **8**, 113-126.

Gatfield, D., and Izaurralde E. (2004) Nonsense-mediated messenger RNA decay is initiated by endonucleolytic cleavage in Drosophila. *Nature*, **429**, 575-578.

Gorman, M.A., Morera, S., Rothwell, D.G., Fortelle, E, Mol, C.D., Tainer, J.A. Hickson, I.D. and Freemont, P.S. (1997) The crystal structure of the human repair endonuclease HAP1 suggests the recognition of extra-helical deoxyribose at DNA abasic sites. *EMBO J*, **16**, 6548-6558.

Gutierrez, R.A., MacIntosh, G.C. and Green, P.J. (1999) Current perspective on mRNA stability in plants: Mutiple levels and mechanisms of control. *Trends Plant Sci*, **4**, 429-438.

Hadi, M.Z., Coleman, M.A., Fidelis, K., Mohrenweiser, H.W. and Wilson, D.M III. (2000) Functional characterization of APE1 variants identified in the human population. *Nucleic Acids Res,* **28**, 3871-3879.

Han, J.Q., Wroblewski, G., Xu, Z., Silverman, R.H. and Barton, D.J. (2004) Sensitivity of hepatitis C virus RNA to the antiviral enzyme ribonuclease L is determined by a subset of efficient cleavage sites. *J Interferon Cytokine Res,* **24**, 664–676.

Hayward, C., Colville, S., Swingler, R.J., Brock, D.J. (1999) Molecular genetic analysis of the APEX nuclease gene in amyotrophic lateral sclerosis. *Neurology*, **52**, 1899-1901.

Hollien, J. and Weissman JS (2006) Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science*, **313**, 104–107.

Houseley, J. and Tollervey D. (2009) The many pathways of RNA degradation. *Cell*, **136**, 763-776.

Hung, R.J., Hall, J., Brennan, P. and Boffetta, P. (2005) Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. *Am J Epidemoil*, **162**, 925-942.

Huntzinger, E., Kashima, I., Fauser, M., Sauliere, J. and Izaurralde, E. (2008) SMG6 is the catalytic endonuclease that cleaves mRNAs containing nonsense codons in metazoans. *RNA*, **14**, 2609-2617.

Ioannidis, P., Kottaridi, C., Dimitriadis, E., Courtis, N., Mahaira, L., Talieri M., Giannopoulos, A., Iliadis, K., Papaioannou, D., Nasioulas, G. and Trangas, T. (2004) Expression of the RNA-binding protein CRD-BP in brain and non-small cell lung tumors. *Cancer Lett*, **209**, 245-250.

Ito, H., Matsuo, K., Hamajima, N., Mitsudomi, T., Sugiura, T., Saito, T., Yasue, T., Lee, K.M., Kang, D., Yoo, K.Y., Sato, S., Ueda. R. and Tajima, K. (2004) Gene-environment interactions between the smoking habit and polymorphisms in the DNA repair genes, APE1 Asp148Glu and XRCC1 Arg399Gln, in Japanese lung cancer risk. *Carcinogenesis*, **25**, 1395-1401.

Izumi, T., Brown, D.B., Naidu, C.V., Bhakat, K.K, MacInnes, M.A., Saito, H., Chen, D.J. and Mitra, S. (2005) Two essential but distinct functions of the mammalian abasic endonuclease. *Proc Natl Acad Sci USA*, **102**, 5739-5743.

Jayaraman, L., Murthy, K., Zhu, C., Curran, T., Xanthoudakis, S. and Prives, C. (1997) Identification of redox/repair protein Ref-1 as a potent activator of p53. *Genes Dev,* **11**, 558-570.

Jiao, L., Bondy, M.L., Hassan, M.M., Wolff, R.A., Evans, D.B., Abbruzzese, J.L. and Li, D. (2006) Selected polymorphisms of DNA repair genes and risk of pancreatic cancer. *Cancer Detect Prev*, **30**, 184-191.

Kaneda, K., Sekiguchi, J. and Shida, T. (2006) Role of the tryptophan residue in the vicinity of the catalytic center of exonuclease III family AP endonucleases: AP site recognition mechanism. *Nucleic Acids Res,* **34**, 1552:1563.

Karginov, F.V., Cheloufi, S., Chong, M.M.W., Stark, A., Smith, A.D., and Hannon, G.J. (2010) Diverse endonucleolytic cleavage sites in the mammalian transcriptome depend upon microRNAs, drosha, and additional nucleases. *Mol Cell*, **38**, 781-778.

Kasahara, M., Osawa, K., Yoshida, K., Miyaishi, A., Osawa, Y., Inoue, N., Tsutou, A., Tabuchi, Y., Tanaka, K., Yamamoto, M., Shimada, E. and Takahashi, J. (2008) Association of MUTYH Gln324His and APEX1 Asp148Glu with colorectal cancer and smoking in a Japanese population. *J Exp Clin Cancer Res*, **27**, 49.

Kelemen, B.R., Klink, T.A., Behlke, M.A., Eubanks, S.R., Leland, P.A. and Raines, R.T. (1999) Hypersensitive substrate for ribonucleases. *Nucleic Acids Res,* **27**, 3696–3701.

Kelley, M.R., Cheng, L., Foster, R., Tritt, R., Jiang, J., Broshears, J. and Koch, M. (2001) Elevated and altered expression of the multifunctional DNA base excision repair and redox enzyme Apel/ref-1 in prostate cancer. *Clin Cancer Res*, **7**, 824-830.

Kim, W.C. 2009. Characterizing the Endoribonuclease Activity of APE1 [M.Sc. Thesis]. Prince George, University of Northern British Columbia.

Kim, B.M., Schultz, L.W. and Raines, R.T. (1999) Variants of ribonuclease inhibitor that resist oxidation. *Protein Sci*, **8**, 430–4.

Kim, W.C., Berquist, B.R., Chohan, M., Uy, C. and Wilson, D.M. (2011) Characterization of the endoribonuclease active site of human apurininic/sprimidinic endonuclease 1. *J Mol Biol*, **411**, 960-971.

Kim, W.C., Ma, C., Kim, S-E., Li, W.M., Wilson, D.M. III., and Lee, C. (2012). Distinct Endoribonuclease Function of Apurinic/Apyrimidinic Endonuclease 1 Variants Identified in the Human Population. Submitted to *J Mol Biol.*

Kim, V.N., Han, J. and Siomi, M.C. (2009) Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol*, **10**, 126–139.

Kim, M.H., Kim, H.B., Acharya, S., Sohn, H.M., Jun, J.Y., Chang, I.Y., You, H.J. (2009) Apel/Ref-1 induces glial cell-derived neurotropic factor (GDNF) responsiveness by upregulating GDNF receptor alpha1 expression. *Mol Cell Biol*, **29**, 2264-2277.

Knight, S.W. and Bass, B.L. (2001) A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. *Science*, **293**, 2269–2271.

Laneve, P., Gioia, U., Ragno, R., Altieri, F., Di Franco, C., Santini, T., Arceci, M., Bozzoni, I. and Caffarelli, E. (2008) The tumor marker human placental protein 11 is an endoribonuclease. *J Biol Chem,* **283**, 34712–34719.

Lebreton, A., Tomecki, R., Dziembowski, A. and Seraphin B. (2008) Endonucleolytic RNA cleavage by a eukaryotic exosome. *Nature,* **456**, 993-996.

Lee, H.C. and Goodman, J.L. (2000) Anaplasma phagocytophilum causes global induction of antiapoptosis in human neutrophils. *Genomics*, **88**, 496-503.

Li, W. M., Barnes T. and Lee C. H. (2010) Endoribonucleases-enzymes gaining spotlight in mRNA metabolism. *FEBS Journal,* **277**, 627-641.

Li, Z., Guan, W., Li, M.X., Zhong, Z.Y., Qian, C.Y., Yang, X.Q., Liao, L., Li, Z.P., Wang, D. (2011) Genetic polymorphism of DNA base-excision repair genes (APE1, OGG1 and XRCC1) and their correlation with risk of lung cancer in a Chinese population. *Arch Med Res*, **42**, 226-234.

Li, C., Hu, Z., Lu, J., Liu, Z., Wang, L.E., El-Naggar, A.K., Sturgis, E.M., Spitz, M.R. and Wei, Q. (2007) Genetic polymorphisms in DNA base-excision repair genes ADPRT, XRCC1, and APE1 and the risk of squamous cell carcinoma of the head and neck. *J Exp Clin Cancer Res*, **28**, 37.

Li, D., Li, Y., Jiao, L., Chang, D.Z., Beinart, G., Wolff, R.A., Evans, D.B., Hassan, M.M., Abbruzzese, J.L. (2007) Effects of base excision repair gene polymorphisms on pancreatic cancer survival. Int J Cancer, **120**, 1746-1754.

Li, .WQ., Zhang, L., Ma, J.L., Zhang, Y., Li, J.Y., Pan, K.F., You, W.C. (2009) Association between genetic polymorphisms of DNA base excision repair genes and evolution of precancerous gastric lesions in a Chinese population. *Carcinogenesis*, **30**, 500-505.

Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L. and Hannon, G.J. (2004) Argonaute2 is the catalytic engine of mammalian RNAi. *Science,* **305**, 1437–1441.

Liu, W., Liang, S.L., Liu, H., Silverman, R. and Zhou, A. (2007) Tumour suppressor function of RNase L in a mouse model. *Eur J Cancer,* **43**, 202–209.

Lo, Y.L., Jou, Y.S., Hsiao, C.F., Chang, G.C., Tsai, Y.H., Su, W.C., Chen, K.Y., Chen, Y.M., Huang, M.S., Hu, C.Y., Chen, C.J. and Hsiung C.A. (2009) A polymorphism in the APE1 gene promoter is associated with lung cancer risk. *Cancer Epidemiol Biomarkers Prev*, **18**, 223-229.

Luo, M. and Kelley, M.R. (2004) Inhibition of the human apurinic/apyrimidinic endonuclease (APE1) repair activity and sensitization of breast cancer cells to DNA alkylating agents with lucanthone. *Anticancer Res*, **24**, 2127-2134.

Lorentzen, E., Basquin, J., Tomecki, R., Dziembowski, A. and Conti, E. (2008) Structure of the active subunit of the yeast exosome core, Rrp44: diverse modes of substrate recruitment in the RNase II nuclease family. *Mol Cell*, **29**, 717–728.

Lu, J., Zhang, S., Chen, D., Wang, H., Wu, W., Wang, X., Lei, Y., Wang, J., Qian, J., Fan, W., Hu, Z., Jin, L., Shen, H., Huang, W., Wei, Q., Lu, D. (2009) Functional characterization of a promoter polymorphism in APE1/Ref-1 that contributes to reduced lung cancer susceptibility. *FASEB J*, **23**, 3459-69.

Maat, W., Beiboer, S.H., Jager, M.J., Luyten, G.P., Gruis, N.A. and van der Velden, P.A. (2008) Epigenetic regulation identifies RASEF as a tumor-suppressor gene in uveal melanoma. *Invest Opthalmol Vis Sci*, **49**, 1291-1218.

Mantha, A.K., Oezguen, N., Bhakat, K.K., Izumi, T., Braun, and W. Mitra, S. (2008) Unusual role of a cysteine residue in substrate binding and activity of human AP-endonuclease 1. *J Mol Biol*, **379**, 28-37.

Martinez, J., Ren, Y.G., Nilsson, P. Ehrenberg, M. and Virtanen A. (2001) The mRNA cap structure simulates rate of poly(A) removal and amplifies processivity of degradation. *J Biol Chem*, **276**, 27923-27929.

Malathi, K., Paranjape, J.M., Ganapathi, R. and Silverman, R.H. (2004) HPC1/RNASEL mediates apoptosis of prostate cancer cells treated with 2′,5′-oligoadenylates, topoisomerase I inhibitors, and tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Res*, **64**, 9144–9151.

McNeill, D.R., Lam, W., DeWeese, T.L., Cheng, Y.C., and Wilson, D.M. III. (2009) Impairment of APE1 function enhances cellular sensitivity to clinically relevant alkylators and antimetabolites. *Mol Cancer Res*, **7**, 897-906.

Misra, R.R., Ratnasinghe, D., Tangrea, J.A., Virtamo, J., Andersen, M.R., Barrett, M., Taylor, P.R., Albanes, D. (2003) Polymorphisms in the DNA repair genes XPD, XRCC1, XRCC3,

and APE/ref-1, and the risk of lung cancer among male smokers in Finland. *Cancer Lett*, **191**, 171-178.

Mitchell, P., Petfalski, E., Shevchenko, A., Mann, M., and Tollervey, D. (1997) The exosome: A conserved eukaryotic RNA processing complex containing multiple 3′→5′ exoribonucleases. *Cell*, **91**, 457–466.

Mello, C.C. and Conte Jr. D. (2004) Revealing the world of RNA interference. *Nature*, **431**, 338-342.

Mercer. T.R., Dinger, M.E., Bracken, C.P., Kolle, G., Szubert, J.M., Korbie, D.J., Askarian-Amiri, M.E., Gardiner, B.B., Goodall, G.J., Grimmond, S.M. and Mattick, J.S. (2011) Regulated post-transcriptional RNA cleavage diversifies the eukaryotic transcriptome. *Genome Res*, **20**, 1639-1650.

Mol, C.D., Izumi, T., Mitra, S. and Tainer, J.A. (2000) DNA-bound structures and mutants reveal abasic DNA binding by APE1 and DNA repair coordination. *Nature*, **403**, 451-156.

Muljo, S.A., Ansel, K.M., Kanellopoulou, C., Livingston, D.M., Rao, A. and Rajewsky, K. (2005) Aberrant T cell differentiation in the absence of Dicer. *J Exp Med*, **202**, 261–269.

Narter, K.F., Ergen, A., Agaçhan, B., Görmüs, U., Timirci, O. and Isbir, T. (2009) Bladder cancer and polymorphisms of DNA repair genes (XRCC1, XRCC3, XPD, XPG, APE1, hOGG1). *Anticancer Res*, **29**, 1389-1393.

Nicholson, A.W. (1999) Function, mechanism and regulation of bacterial ribonucleases. *FEMS Microbiol Rev*, **23**, 371-390.

Oezgeun, N., Schein, C.H., Peddi, S.R., Power, T.D., Izumi, T., and Braun, W. (2007) A "moving metal mechanism" for substrate cleavage by the DNA repair endonuclease APE-1. *Proteins*, **68**, 313-323.

Olkowiski, Z.L. (1998) Mutatant AP endonuclease in patients with amytrophic lateral sclerosis. *Neuroreport*, **9**, 239-242.

Ordway, J.M., Eberhart, D. and Curran, T. (2003) Cysteine 64 of Ref-1 Is Not Essential for Redox Regulation of AP-1 DNA Binding. *Mol Cell Biol*, **23**, 4257–4266.

Palli, D., Polidoro, S., D'Errico, M., Saieva, C., Guarrera, S., Calcagnile, A.S., Sera, F., Allione, A., Gemma, S., Zanna, I., Filomena, A., Testai, E., Caini, S., Moretti, R., Gomez-Miguel, M.J., Nesi, G., Luzzi, I., Ottini, L., Masala, G., Matullo, G. and Dogliotti, E. (2010) Polymorphic DNA repair and metabolic genes: a multigenic study on gastric cancer. *Mutagenesis*, **25**, 569-575.

Pardini, B., Naccarati, A., Novotny, J., Smerhovsky, Z., Vodickova, L., Polakova, V., Hanova, M., Slyskova, J., Tulupova, E., Kumar, R., Bortlik, M., Barale, R., Hemminki, K. and Vodicka, P. (2008) DNA repair genetic polymorphisms and risk of colorectal cancer in the Czech Republic. *Mutat Res*, **638**, 146-153.

Parker, R. and Song, H. (2004) The enzymes and control of eukaryotic mRNA turnover. *Nat Struct Mol Biol,* **11**, 121-127.

Peng, Y., Liu, X. and Schoenberg, D.R. (2008) The 90-kDa heat shock protein stabilizes the polysomal ribonuclease 1 mRNA endonuclease to degradation by the 26S proteasome. *Mol Biol Cell*, **19**, 546–552.

Piazza, O., Sirén, A.L. and Ehrenreich, H. (2004) Soccer, neurotrauma and amyotrophic lateral sclerosis: is there a connection? *Curr Med Res Opin*, **20**, 505-508.

Ponniah, K., Loo, T.S., Edwards, P.J., Pascal, S.M., Jameson, G.B. and Norris GE. (2010) The production of soluble and correctly folded recombinant bovine beta-lactoglobulin variants A and B in Escherichia coli for NMR studies. *Protein Expr Purif*, **70**, 283-289.

Qing, Y., Wang, D., Lei, X., Xiang, D.B., Li, M.X., Li, Z.P. and Shan, J.L. (2009) The expression of APE1 and its correlation with prognostic significance after 252Cf radiotherapy in cervical cancer. *Sichuan Da Xue Xue Bao Yi Xue Ban*, **40**, 125-129.

Raghavan, A., Ogilvie, R.L., Reilly, C., Abelson, M.L., Raghavan, S., Vasdewani, J., Krathwohl, M. and Bohjanen, P.R. (2002). Genome-wide analysis of mRNA decay in resting and activated primary human T lymphocytes. *Nucleic Acids Res*, **30**, 5529-5538.

Ron, D. and Hubbard, S.R. (2008) How IRE1 reacts to ER stress. *Cell,* **132**, 24–26.

Ross, J. (1995) mRNA stability in mammalian cells. *Microbiol Rev*, **59**, 423-450.

Saxena, S.K., Rybak, S.M., Davey, R.T. Jr., Youle, R.J. and Ackerman, E.J. (1992) Angiogenin is a cytotoxic, tRNA-specific ribonuclease in the RNase A superfamily. *J Biol Chem*, **267**, 21982–21986.

Schaeffer, D., Tsanova, B., Barbas, A., Reis, F.P., Dastidar, E.G., Sanchez-Rotunno, M., Arraiano, C.M. and van Hoof, A. (2009) The exosome contains domains with specific endoribonuclease, exoribonuclease and cytoplasmic mRNA decay activities. *Nat Struct Mol Biol*, **16**, 56-62.

Schneider, C., Leung, E., Brown, J. and Tollervey, D. (2009) The N-terminal PIN domain of the exosome subunit Rrp44 harbors endonuclease activity and tethers Rrp44 to the yeast core exosome. *Nucleic Acids Res*, **37**, 1127–1140.

Shintani, M., Tada, M., Kobayashi, T., Kajiho, H., Kontani, K. and Katada T. (2007) Characterization of Rab45/RASEF containing EF-hand domain and a coiled-coil motif as a self-associating GTPase. *Biochem Biophys Commun*, **357**, 661-667.

Silverman, R.H. (2003) Implications for RNase L in prostate cancer biology. *Biochemistry*, **42**, 1805-1812.

Smith, B.D. and Raines, R.T. (2006) Genetic selection for critical residues in ribonucleases. *J Mol Biol*, **362**, 459-478.

Smith, B.D and Raines, R.T. (2008) Genetic selection for peptide inhibitors of angiogenin. *Protein End Des Sel*, **21**, 289-194.

Srivastava, D.K., Berg, B.J., Prasad, R., Molina, J.T., Beard, W.A., Tomkinson, A.E. and Wilson, S.H. (1998) Mammalian Abasic Site Base Excision Repair. Identification of the reaction sequence and rate-determining steps. *J Biol Chem*, **273**, 21203-21209.

Stefanizzi, I. and Canete-Soler, R. (2007) Coregulation of light neurofilament mRNA by poly(A)-binding protein and aldolase C: implications for neurodegeneration. *Brain Res*, **1139**, 15–28.

Steinman, R.A. (2007) mRNA stability control: a clandestine force in normal and malignant hematopoiesis. *Leukemia*, **21**, 1158-1171.

Tell, G. Damante, G., Caldwell, D., and Kelley, M.R. (2005) The intracellular localization of APE1/Ref-1: more than a passive phenomenon? *Antioxid Redox Signal*, **7**, 367-384.

Terry, P.D., Umbach, D.M. and Taylor, J.A. (2006) APE1 genotype and risk of bladder cancer: evidence for effect modification by smoking. *Int J Cancer*, **118**, 3170-3173.

Thomsen, S., Anders, S., Janga, S.C., Huber, W. and Alonso, C.R. Genome-wide analysis of mRNA decay patterns during early Drosophila development. *Genome Biol*, **11**, R39.

Tomecki, R. and Dziembowski A. (2010) Novel endoribonucleases as central players in various pathways of eukaryotic RNA metabolism. *RNA*, **16**, 1692-1724.

Tomkins, J., Dempster, S., Banner, S.J., Cookson, M.R. and Shaw, P.J. (2000) Screening of AP endonuclease as a candidate gene for amyotrophic lateral sclerosis (ALS). Neuroreport, **11**, 1695-1697.

Tourriere, H., Gallouzi, I.E., Chebli, K., Capony, J.P., Mouaikel, J., van der Geer, P. and Tazi, J. (2001) RasGAP-associated endoribonuclease G3Bp: selective RNA degradation and phosphorylation-dependent localization. *Mol Cell Biol*, **21**, 7747–7760.

Tse, D., Zhai, R., Zhou, W., Heist, R.S., Asomaning, K., Su, L., Lynch, T.J., Wain, J.C., Christiani, D.C. and Liu, G. (2008) Polymorphisms of the NER pathway genes, ERCC1 and XPD are associated with esophageal adenocarcinoma risk. *Cancer Causes Control*, **19**, 1077-1083,

Vascotto, C., Cesaratto, L., Zeef, L.A., Deganuto, M., D'Ambrosio, C., Scaloni, A., Romanello, M., Damante, G., Taglialatela, G., Delneri, D., Kelley, M.R., Mitra, S., Quadrifoglio, F. and Tell, G. (2009) Genome-wide analysis and proteomic studies reveal APE1/Ref-1 multifunctional role in mammalian cells. *Proteomics*, **9**, 1058-1074.

Vascotto, C., Fantini, D., Romanello, M., Cesaratto, L., Deganuto, M., Leonardi, A., Radicella, J.P., Kelley, M.R., D'Ambrosio, C., Scaloni, A., Quadrifoglio, F. and Tell G. (2009) APE1/Ref-1 interacts with NPM1 within nucleoli and plays a role in the rRNA quality control process. *Mol Cell Biol,* **29**, 1834-1854.

Walker, L.J., Robson, C.N., Black, E., Gillespie, D., and Hickson, I.D. (1993) Identification of residues in the human DNA repair enzyme HAP1 (Ref-1) that are essential for redox regulation of Jun DNA binding. *Mol Cell Biol,***13**,5370-5376.

Wang, B., Wang, D., Huang, G, Zhang, C., Xu, D.H., Zhou, W. (2010) XRCC1 polymorphisms and risk of coleorectal cancer: a meta-analysis. *Int J Colorectal Dis*, **25**, 313-321.

Wang, M. and Cohen, S.N. (1994) ard-1: a human gene that reverses the effects of temperature-sensitive and deletion mutations in the Escherichia coli rne gene and encodes an activity producing RNase E-like cleavages. *Proc Natl Acad Sci USA*, **91**, 10591–10595.

Wang, D., Luo, M., Kelley, M.R. (2004) Human apurinic endonuclease 1 (APE1) expression and prognostic significance in osteosarcoma: enhanced sensitivity of osteosarcoma to DNA damaging agents using silencing RNA APE1 expression inhibition. *Mol Cancer Ther*, **3**, 679-686.

Wang, A., Su, Y., Wang, S., Shen, M., Chen, F., Chen, M., Ran, X., Cheng, T. and Wang, J. (2010) High efficiency preparation of bioactive human alpha-defensin 6 in Escherichia coli Origami(DE3)pLysS by soluble fusion expression. *Appl Microbiol Biotechnol*, **87**, 1935-1942.

Wang, M., Qin, C., Zhu, J., Yuan, L., Fu, G., Zhang, Z., Yin, C. (2010) Genetic variants of XRCC1, APE1, and ADPRT genes and risk of bladder cancer. *DNA Cell Biol*, **29**, 303-311.

Wang, D., Xiang, D.B., Yang, X.Q., Chen, L.S., Li, M.X., Zhong, Z.Y., Zhang, Y.S. (2009) APE1 overexpression is associated with cisplatin resistance in non-small cell lung cancer and targeted inhibition of APE1 enhances the activity of cisplatin in A549 cells. *Lung Cancer*, **66**, 298-304.

Wang, D., Zhong, Z.Y., Li, M.X., Xiang, D.B., and Li, Z.P. (2007) Vector-based Ape1 small interfering RNA enhances the sensitivity of human osteosarcoma cells to endostatin in vivo. *Cancer Sci*, **98**, 1993-2001.

Weiss, J.M., Goode, E.L., Ladiges, W.C. and Ulrich, C.M. (2005) Polymorphic variation in hOGG1 and risk of cancer: a review of the functional and epidemiologic literature. *Mol Carcinog*, **42**, 127-141.

Wen-Bin, M., Wei, W., Yu-Lan, Q., Fang, J., Zhao-Lin, X. (2009) Micronucleus occurrence related to base excision repair gene polymorphisms in Chinese workers occupationally exposed to vinyl chloride monomer. *J Occup Environ Med*, **51**, 578-585.

Xanthoudakis, S., Miao, G., Wang, F., Pan, Y.C, and Curran, T. (1992) Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme. *EMBO J*, **11**, 323-3353.

Xiang, D.B., Chen, Z.T., Wang, D., Li M.X., Xie, J.Y., Zhang, Y.S., Qing, Y., Li, Z.P. and Xie, J. (2008) Chimeric adenoviral vector Ad5/F35-mediated APE1 siRNA enhances sensitivity of human colorectal cancer cells to radiotherapy in vitro and in vivo. *Cancer Gene Ther*, **15**, 625-635.

Yang, F. and Schoenberg, D.R. (2004) Endonuclease-mediated mRNA decay involves the selective targeting of PMR1 to polyribosome-bound substrate mRNA. *Mol Cell,* **14**, 435–445.

Yoo, D.G., Song, Y.J., Cho, E.J., Lee, S.K., Park, J.B., Yu, J.H., Lim, S.P., Kim, J.M. and Jeon B.H. (2008) Alteration of APE1/ref-1 expression in non-small cell lung cancer: the implications of impaired extracellular superoxide dismutase and catalase antioxidant systems. *Lung Cancer*, 60, 277-84.

Zhang, H., Cowan-Jacob, S.W., Simonen, M., Greenhalf, W., Heim, J. and Meyhack, B. (2000) Structural basis of BFL-1 for its interaction with BAX and its anti-apoptotic action in mammalian and yeast cells. *J Biol Chem*, **275**, 11092-11099.

Zienolddiny, S., Campa, D., Lind, H., Ryberg, D., Skaug, V., Stangeland, L., Phillips, D.H., Canzian, F. and Haugen, A. (2006) Polymorphisms of DNA repair genes and risk of non-small cell lung cancer. *Carcinogenesis,* **27**, 560-567.

Zekri, L., Chebli, K., Tourriere, H., Nielsen, F.C., Hansen, T.V., Rami, A. and Tazi, J. (2005) Control of fetal growth and neonatal survival by the RasGAP-associated endoribonuclease G3BP. *Mol Cell Biol,* **25**, 8703–8716.

Zhou, K., Hu, D., Lu, J., Fan, W., Liu, H., Chen, H., Chen, G., Wei, Q., Du, G., Mao, Y., Lu, D., Zhou, L. (2011) A genetic variant in the APE1/Ref-1 gene promoter -141T/G may modulate risk of glioblastoma in a Chinese Han population. *BMC Cancer*, **11**, 104.

Zhou, A., Paranjape, J., Brown, T.L., Nie, H., Naik, S., Dong, B., Chang, A., Trapp, B., Fairchild, R., Colmenares, C. and Silverman, R.H. (1997) Interferon action and apoptosis are defective in mice devoid of 2',5'-oligoadenylate-dependent RNase L. *EMBO J*, **16**, 6355–6363.

Zuo, Y. and Deutscher M.P. (2001) Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. *Nucleic Acids Res*, **29**, 1017-1026.